NT69, A NOVEL NUCLEOSIDE TRANSPORTER FAMILY MEMBER AND USES THEREFOR

Background of the Invention

[0001] Nucleoside transporters, present in organellar membranes and in the plasma membranes of most cell types, are integral membrane proteins that mediate the uptake and release of nucleosides and nuleoside analog drugs.

[0002] Mammalian nucleoside transporters are divided into two functionally and structurally distinct families. See, Cass et al. *Pharm Biotechnol.* (1999) 12:313-52. Concentrative nucleoside transporters (CNT) mediate Na^+-dependent transport of nucleosides against their concentration gradients and are predicted to possess 8 to 14 transmembrane domains. CNT's are classified by their substrate selectivity for pyrimidines (CNT1 transporters), purines (CNT2 transporters), or both. Equilibrative nucleoside transporters (ENT) function by facilitated diffusion and exhibit broad substrate specificity for purine and pyrimidine nucleosides. Two broad subtypes of ENTs are recognized based on their sensitivity (ENT1) or resistance (ENT2) to inhibition by nitrobenzylmercatopurine ribonucleoside (NBMPR). ENT's are predicted to possess 11 transmembrane domains.

[0003] Nucleoside transporters regulate cellular adenosine concentrations, thus profoundly affecting many physiological processes, including metabolism, cell proliferation, adenosine-mediated regulation of neurotransmission and platelet aggregation, and regulation of vascular tone. Baldwin et al. *Mol. Med. Today* (1999) 5:216-24. Furthermore, these transporters are critical for the cellular uptake of potent cytotoxic nucleoside analogues that are broadly used clinically as anticancer and antiviral agents Pastor-Anglada et al. *Trends Pharmacol. Sci.* (1998) 19:424-30. In addition, ENTs are pharmacologic targets for coronary vasodilator drugs. Therefore, the identification of novel nucleotide transporters is important for the treatment of human disease.

Summary of the Invention

[0004] The present invention is based, in part, on the discovery of a novel nucleoside transporter family member, referred to herein as "NT69". The nucleotide sequence of a cDNA encoding NT69 is shown in SEQ ID NO:1 (Figures 1A-1B), and the amino acid

sequence of an NT69 polypeptide is shown in SEQ ID NO:2 (Figures 1A-1B). In addition, the nucleotide sequence of the coding region is depicted in SEQ ID NO:3 (Figures 1A-1B).

[0005] Accordingly, in one aspect, the invention features a nucleic acid molecule which encodes an NT69 protein or polypeptide, e.g., a biologically active portion of the NT69 protein. In a preferred embodiment the isolated nucleic acid molecule encodes a polypeptide having the amino acid sequence of SEQ ID NO:2. In other embodiments, the invention provides isolated NT69 nucleic acid molecules having the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, or the sequence of the DNA insert of the plasmid deposited with ATCC® Accession Number PTA-2533. In still other embodiments, the invention provides nucleic acid molecules (e.g., naturally occurring allelic variants) that are substantially identical to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, or the sequence of the DNA insert of the plasmid deposited with ATCC® Accession Number PTA-2533. In other embodiments, the invention provides a nucleic acid molecule which hybridizes under stringent hybridization conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3, or the sequence of the DNA insert of the plasmid deposited with ATCC® Accession Number PTA-2533, wherein the nucleic acid encodes a full length NT69 protein or an active fragment thereof.

[0006] In a related aspect, the invention further provides nucleic acid constructs which include an NT69 nucleic acid molecule described herein. In certain embodiments, the nucleic acid molecules of the invention are operatively linked to native or heterologous regulatory sequences. Also included, are vectors and host cells containing the NT69 nucleic acid molecules of the invention, e.g., vectors and host cells suitable for producing NT69 nucleic acid molecules and polypeptides.

[0007] In another related aspect, the invention provides nucleic acid fragments suitable as primers or hybridization probes for the detection of NT69-encoding nucleic acids.

[0008] In still another related aspect, isolated nucleic acid molecules that are antisense to an NT69 encoding nucleic acid molecule are provided.

[0009] In another aspect, the invention features, NT69 polypeptides, and biologically active or antigenic fragments thereof that are useful, e.g., as reagents or targets in assays applicable to treatment and diagnosis of NT69 mediated or related disorders. In another embodiment, the invention provides NT69 polypeptides having an NT69 activity. Preferred polypeptides are NT69 proteins including at least one nucleoside transporter signature domain, and, preferably, having an NT69 activity, e.g., an NT69 activity as described herein.

[0010] In other embodiments, the invention provides NT69 polypeptides, e.g., an NT69 polypeptide having the amino acid sequence shown in SEQ ID NO:2; the amino acid sequence encoded by the cDNA insert of the plasmid deposited with ATCC® Accession Number 2533; an amino acid sequence that is substantially identical to the amino acid sequence shown in SEQ ID NO:2; or an amino acid sequence encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3, or the sequence of the DNA insert of the plasmid deposited with ATCC® Accession Number PTA-2533, wherein the nucleic acid encodes a full length NT69 protein or an active fragment thereof.

[0011] In a related aspect, the invention further provides nucleic acid constructs which include an NT69 nucleic acid molecule described herein.

[0012] In a related aspect, the invention provides NT69 polypeptides or fragments operatively linked to non-NT69 polypeptides to form fusion proteins.

[0013] In another aspect, the invention features antibodies and antigen-binding fragments thereof, that react with, or more preferably specifically bind NT69 polypeptides.

[0014] In another aspect, the invention provides methods of screening for compounds that modulate the expression or activity of the NT69 polypeptides or nucleic acids. Such modulators are candidate compounds for treatment of disorders associated with aberrant expression or activity of NT69.

[0015] In still another aspect, the invention provides a process for modulating NT69 polypeptide or nucleic acid expression or activity, e.g., using the screened compounds. In certain embodiments, the methods involve treatment of conditions related to aberrant activity or expression of the NT69 polypeptides or nucleic acids, such as conditions involving aberrant or deficient cell growth, e.g., cancer; aberrant or deficient neurotransmission, e.g., dementias or opiate addiction; aberrant or deficient vascular tone, e.g., vascular disease and hypertension; aberrant or inappropriate weight gain, metabolic rate, or fat deposition, e.g, anorexia, bulimia, obesity, diabetes, or hyperlipidemia; aberrant or inappropriate pain.

[0016] In still another aspect, the invention provides methods of identifying a subject at risk for a disorder of fat cell proliferation or metabolism comprising detecting, in a tissue of the subject, misexpression of NT69.

[0017] In a further aspect, the invention provides methods of identifying a subject at risk for a disorder of fat cell proliferation or metabolism comprising detecting, in a tissue of the

subject, the presence or absence of a mutation which affects the expression or activity of NT69.

[0018] In yet another aspect, the invention provides methods of identifying a subject at risk for a disorder of pain comprising detecting, in a tissue of the subject, misexpression of NT69.

[0019] In a further aspect, the invention provides methods of identifying a subject at risk for a disorder of pain comprising detecting, in a tissue of the subject, the presence or absence of a mutation which affects the expression or activity of NT69.

[0020] The invention also provides assays for determining the activity of or the presence or absence of or the level of NT69 polypeptides or nucleic acid molecules in a biological sample, including for disease diagnosis.

[0021] In further aspect the invention provides assays for determining the presence or absence of a genetic alteration in an NT69 polypeptide or nucleic acid molecule, including for disease diagnosis.

[0022] Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

<u>Brief Description of the Drawings</u>

[0023] *Figures 1A-1B* depicts a cDNA sequence (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human NT69. The methionine-initiated open reading frame of human NT69 (without the 5' and 3' untranslated regions) extends from nucleotide 52 to nucleotide 1477 (shown also as coding sequence SEQ ID NO:3).

[0024] *Figure 2* depicts a hydropathy plot of human NT69. Relative hydrophobic residues are shown above the dashed horizontal line, and relative hydrophilic residues are below the dashed horizontal line. The cysteine residues (cys) are indicated by short vertical lines just below the hydropathy trace. The numbers corresponding to the amino acid sequence of human NT69 are indicated. Polypeptides of the invention include fragments which include: all or part of a hydrophobic sequence, i.e., a sequence above the dashed line, e.g., the sequence of from about amino acid residue 50 to about amino acid residue 70, or from about amino acid residue 360 to about amino acid residue 380, of SEQ ID NO:2; all or part of a hydrophilic sequence, i.e., a sequence below the dashed line, e.g., the sequence of from about amino acid residue 205 to about amino acid residue 225 of SEQ ID NO:2; a

sequence which includes a Cys, or a glycosylation site. Polypeptides of the invention also include fragments which include one or more of the nine putative transmembrane domains.

[0025] *Figure 3A* depicts an alignment of the nucleoside transporter signature domain of NT69 with a consensus amino acid sequence (PF01733) derived from a hidden Markov model from PFAM. The upper sequence is the consensus amino acid sequence (SEQ ID NO:4), while the lower amino acid sequence corresponds to amino acids 115 to 452 of SEQ ID NO:2.

[0026] *Figure 3B* depicts a BLAST alignment of the nucleoside transporter domain of human NT69 with a consensus amino acid sequence derived from a ProDomain No. 4098 (Release 1999.2; http://www.toulouse.inra.fr/prodom.html). The lower sequence is the consensus amino acid sequence (SEQ ID NO:5), while the upper amino acid sequence corresponds to the nucleoside transporter domain of human NT69, about amino acids 127 to 448 of SEQ ID NO:2.

[0027] *Figure 4* depicts a graph illustrating the relative levels of NT69 mRNA expression in normal human tissues. Tissue samples are (1) artery, (2) vein, (3) aortic smooth muscle cells, (4) coronary smooth muscle cells, (5) static human umbilical vein endothelial cells, (6) shear human umbilical vein endothelial cells, (7) heart, (8) heart (congestive heart failure), (9) kidney, (10) skeletal muscle, (11) normal adipose tissue, (12) pancreas, (13) primary osteoblasts, (14) differentiated osteoblasts, (15) normal skin, (16) normal spinal cord, (17) normal brain cortex, (18) hypothalamus, (19) nerve, (20) dorsal root ganglion, (21) glial cells (astrocytes), (22) glioblastoma, (23) breast, (24) breast tumor, (25) ovary, (26) ovary tumor, (27) prostate, (28) prostate tumor, (29) epithelial cells (prostate), (30) colon, (31) colon tumor, (32) lung, (33) lung tumor, (34) lung chronic obstructive pulmonary disease, (35) colon (inflammatory bowel disease), (36) liver, (37) liver fibrosis, (38) dermal cells (fibroblasts), (39) spleen, (40) tonsil, (41) lymph node, (42) resting peripheral blood mononuclear cells, (43) skin decubitus, (44) synovium, (45) bone marrow-mononuclear cells, (46) activated peripheral blood mononuclear cells.

[0028] *Figure 5* depicts a bar graph illustrating the relative levels of the rat ortholog of NT69 mRNA expression in dorsal root ganglion (DRG) taken from rats in three models of pain. CCI is a chronic constriction injury. DRG RNA was obtained on the days 3, 7, 10, and 28 following surgery, as indicated in parentheses. CFA is a model of inflammatory pain. DRG RNA was obtained 1, 3, 7, 10, 14, and 28 days following treatment, as indicated in parentheses. AXT is a model of chronic neuropathic pain. DRG RNA was obtained 1, 3, 7,

and 14 days following surgery, as indicated in parentheses. Each bar represents the pooled RNA from three rats.

[0029] *Figure 6* depicts a bar graph illustrating the relative levels of the rat ortholog of NT69 mRNA expression in spinal cord (SC) taken from rats in the three models of pain described for Figure 5.

Detailed Description

[0030] The human NT69 sequence (Figures 1a-1b; SEQ ID NO:1), which is approximately 2625 nucleotides long including untranslated regions, contains a predicted methionine-initiated coding sequence of about 1425 nucleotides (nucleotides 52 to 1477 of SEQ ID NO:1; nucleotides 1 to 1425 of SEQ ID NO:3). The coding sequence encodes a 475 amino acid protein (SEQ ID NO:2).

[0031] Human NT69 contains the following regions or other structural features:

a predicted nucleoside transporter domain located at about amino acids 115 to 452 of SEQ ID NO:2;

a predicted signal peptide from amino acid 1 to 37, which when cleaved gives a predicted mature protein of 438 amino acids, from amino acid 38 to 475 of SEQ ID NO:2;

two predicted N-glycosylation sites (PS00001) located from about amino acid 341 to about 344, and from about amino acid 468 to about 471, of SEQ ID NO:2;

one predicted cAMP- and cGMP-dependent protein kinase site (PS00004) located from about amino acid 141 to about 144 of SEQ ID NO:2;

six predicted protein kinase C phosphorylation sites (PS00005) located from about amino acid 6 to about 8, from about amino acid 207 to about 209, from about amino acid 213 to about 215, from about amino acid 251 to about 253, from about amino acid 283 to about 285, and from about amino acid 421 to about 423, of SEQ ID NO:2;

three predicted casein kinase II sites (PS00006) located from about amino acid 35 to about 38, from about amino acid 96 to about 99, and from about amino acid 251 to about 254, of SEQ ID NO:2;

ten predicted N-myristoylation sites (PS00008) located from about amino acid 2 to about 7, from about amino acid 21 to about 26, from about amino acid 146 to about 151, from about amino acid 155 to about 160, from about amino acid 219 to about 224, from about amino acid 262 to about 267, from about amino acid 402 to about 407, from about amino acid

429 to about 434, from about amino acid 439 to about 444, and from about amino acid 469 to about 474, of SEQ ID NO:2; and

nine transmembrane domains predicted by MEMSAT located from about amino acid 14 to about 30, from about amino acid 49 to about 72, from about amino acid 81 to about 102, from about amino acid 177 to about 197, from about amino acid 297 to about 319, from about amino acid 331 to about 355, from about amino acid 362 to about 381, from about amino acid 396 to about 418, and from about amino acid 434 to about 453, of SEQ ID NO:2.

[0032] A plasmid containing the nucleotide sequence encoding human NT69 was deposited with American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, VA 20110-2209, on October 3, 2000 and assigned Accession Number PTA-2533. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit was made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

[0033] The NT69 protein contains a significant number of structural characteristics in common with members of the nucleoside transporter family, particularly the equilibrative nucleoside transporter (ENT) subfamily . The term "family" when referring to the protein and nucleic acid molecules of the invention means two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin as well as other distinct proteins of human origin, or alternatively, can contain homologues of non-human origin, e.g., rat or mouse proteins. Members of a family can also have common functional characteristics.

[0034] An NT69 polypeptide can include a "nucleoside transporter domain" or regions homologous with a "nucleoside transporter domain."

[0035] As used herein, the term "nucleoside transporter domain" includes an amino acid sequence of about 200 to about 500 amino acid residues in length and having a bit score for the alignment of the sequence to the nucleoside transporter domain (PF01733) of at least 50. Preferably, a nucleoside transporter domain includes at least about 250 to about 450 amino acids, more preferably about 300 to about 400 amino acid residues, and has a bit score for the alignment of the sequence to the nucleoside transporter domain (PF01733) of at least 75 or greater. For general information regarding PFAM identifiers, PS prefix and PF prefix

domain identification numbers, refer to Sonnhammer et al. (1997) *Protein* 28:405-420 and http://www.psc.edu/general/ software/packages/pfam/pfam.html. An alignment of the nucleoside transporter domain (amino acids 115 to 452 of SEQ ID NO:2) of human NT69 with a consensus amino acid sequence of a nucleoside transporter domain (PFAM Accession PF01733) derived from a hidden Markov model is depicted in Figure 3.

[0036] In a preferred embodiment NT69 polypeptide or protein has a "nucleoside transporter domain" or a region which includes at least about 200 to about 500, more preferably about 250 to about 450 or about 290 to about 400 amino acid residues and has at least about 60%, 70%, 80%, 90%, 95%, 99%, or 100% homology with a "nucleoside transporter domain," e.g., the nucleoside transporter domain of human NT69 (e.g., residues 115 to 452 of SEQ ID NO:2).

[0037] To identify the presence of a "nucleoside transporter" domain in an NT69 protein sequence, and make the determination that a polypeptide or protein of interest has a particular profile, the amino acid sequence of the protein can be searched against a database of HMMs (e.g., the Pfam database, release 2.1) using the default parameters (http://www.sanger.ac.uk/Software/Pfam/HMM_search). For example, the hmmsf program, which is available as part of the HMMER package of search programs, is a family specific default program for MILPAT0063 and a score of 15 is the default threshold score for determining a hit. Alternatively, the threshold score for determining a hit can be lowered (e.g., to 8 bits). A description of the Pfam database can be found in Sonhammer et al. (1997) *Proteins* 28(3):405-420 and a detailed description of HMMs can be found, for example, in Gribskov et al. (1990) *Meth. Enzymol.* 183:146-159; Gribskov et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:4355-4358; Krogh et al. (1994) *J. Mol. Biol.* 235:1501-1531; and Stultz et al. (1993) *Protein Sci.* 2:305-314, the contents of which are incorporated herein by reference. A search was performed against the HMM database resulting in the identification of a "nucleoside transporter domain" domain in the amino acid sequence of human NT69 at about residues 115 to 452 of SEQ ID NO:2 (see Figure 3A). The nucleoside transporter domain is homologous to ProDomain No. 4098 (Release 1999.2; http://www.toulouse.inra.fr/prodom.html). Figure 3B depicts an alignment of the nucleoside transporter domain of human NT69 with a consensus nucleoside transporter amino acid sequence derived from ProDomain No. 4098.

[0038] An NT69 polypeptide can further include at least one "transmembrane domain" or regions homologous to a "transmembrane domain."

[0039] As used herein, the term "transmembrane domain" includes an amino acid sequence of at least about 15 amino acid residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes about 20 amino acid residues and spans the plasma membrane. Transmembrane domains are rich in hydrophobic residues, and typically have an alpha-helical structure. In a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, 95% or more of the amino acids of a transmembrane domain are hydrophobic, e.g., leucines, isoleucines, tyrosines, or tryptophans. Transmembrane domains are described in, for example, Zagotta et al. (1996) *Annual Rev. Neurosci.* 19: 235-263, the contents of which are incorporated herein by reference. Amino acid residues 14 to 30, 49 to 72, 81 to 102, 177 to 197, 297 to 319, 331 to 355, 362 to 381, 396 to 418, and 434 to 453 of the NT69 protein (SEQ ID NO:2) are predicted to be transmembrane domains (see Figure 2). Accordingly, NT69 proteins having at least 50-60% homology, preferably about 60-70%, more preferably about 70-80%, or about 80-90% homology with at least one transmembrane domain of human NT69 are within the scope of the invention.

[0040] In a preferred embodiment, NT69 protein has nine "transmembrane domains" or a region which includes at least about 15, more preferably about 20 amino acid residues and has at least about 50%, 60%, 70%, 80%, 90%, 95%, 99%, or 100% homology with a "transmembrane domain," e.g., the transmembrane domains of NT69 protein.

[0041] As the NT69 polypeptides of the invention may modulate NT69-mediated activities, they may be useful as of for developing novel diagnostic and therapeutic agents for NT69-mediated or related disorders, as described below.

[0042] As used herein, a "NT69 activity", "biological activity of NT69" or "functional activity of NT69", refers to an activity exerted by an NT69 protein, polypeptide or nucleic acid molecule on e.g., an NT69-responsive cell or on an NT69 substrate, e.g., a protein substrate, as determined *in vivo* or *in vitro*. In one embodiment, an NT69 activity is a direct activity, such as an association with an NT69 target molecule. A "target molecule" or "binding partner" is a molecule with which an NT69 protein binds or interacts in nature. In an exemplary embodiment, a binding partner is an NT69 substrate, e.g., a nucleoside. An NT69 activity can also be an indirect activity, e.g., a cellular signaling activity mediated by interaction of the NT69 protein with an NT69 substrate. For example, the NT69 protein of the present invention can have one or more of the following activities: (1) it can catalyze the transport of a nucleoside or nucleoside analog across a cell membrane; (2) it can modulate DNA synthesis; or (3) it can modulate cellular proliferation and/or differentiation of a cell or

tissue, e.g., a tissue in which an NT69 protein is expressed, e.g., fat tissue or neurological, e.g., dorsal root ganglion, superior cervical ganglion, spinal cord, or brain tissue.

[0043] Based on the above-described sequence similarities, the NT69 molecules of the present invention are predicted to have similar biological activities as nucleoside transporter family members.

[0044] Altered nucleoside transporter activity has been reported in cancerous conditions. For example, nucleoside transporter expression is decreased in hepatocarcinomas. Dragan et al. (2000) *Hepatology* 32:239-46. In multiple myeloma patients with progressive disease, there is increased nucleoside transporter expression compared to those with stable disease. Petersen et al. (1994) *Leuk. Lymphoma* 13:491-9. Overexpression of nucleoside transporters has also been found in human breast, liver, stomach and colorectal tumor tissues. Goh et al. (1995) *Anticancer Res.* 15:2575-9. Therefore, modifying expression of a nucleoside transporter can be expected to reduce or eliminate cancer cell growth. Accordingly, the NT69 molecules can act as novel diagnostic targets and therapeutic agents for controlling malignant disorders, e.g., cancers.

[0045] Altered nucleoside transporter activity has also been reported in conditions related to neurotransmission and vascular tone. The adenosine transporter, for example, is up-regulated in opiate tolerant mice (Kaplan et al. (1997) *Brain Res.* 763:215-20), while nucleoside transporters are down-regulated in umbilical vein endothelial cells in diabetic pregnancies (Sobrevia et al. (1994) *Am. J. Physiol* 267:C39-47). Accordingly, the NT69 molecules can act as novel diagnostic targets and therapeutic agents for controlling disorders of neurotransmission and vascularization as well. Examples of such disorders include, but are not limited to, e.g., dementias, opiate addiction, and vascular disease.

[0046] The NT69 mRNA is expressed highest in brain tissue and fat tissue (Figure 4). It is thus likely that NT69 molecules of the present invention may be involved in disorders characterized by aberrant activity of these cells. Examples of disorders associated with brain tissue include, but are not limited to, neurodegenerative disorders, e.g., Alzheimer's disease, Huntington's disease, Parkinson's and other Lewy diffuse body diseases, multiple sclerosis, amyothrophic lateral sclerosis, progressive supranuclear palsy, epilepsy, and Jakob-Creutzfieldt disease; psychiatric disorders, e.g., depression, schizophrenic disorders, Korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, e.g., amnesia or age-related memory loss; and neurological disorders, e.g., migraine and pain, e.g., acute or chronic pain. Examples of pain disorders include, but are

not limited to, the exaggerated pain response elicited during various forms of tissue injury, *e.g.*, inflammation, infection, and ischemia, usually referred to as hyperalgesia (described in, for example, Fields, H.L. (1987) *Pain*, New York:McGraw-Hill). Chronic pain includes chronic intractable pain, e.g., pain associated with carcinomatosis; invasion or compression syndromes due to cancer; mental illness; neurologic disorders such as neuralgias, phantom limb pain, nerve entrapment syndromes, spinal cord damage, myofascial syndromes, and thalamic syndrome pain. Other examples include pain associated with musculoskeletal disorders, *e.g.*, joint pain; tooth pain; headaches; or pain associated with surgery, rheumatoid arthritis, viral infection, allergic reaction, asthma, chronic pain, chronic pancreatitis, somatoform disorders, fibromyalgia syndrome, and the like.

[0047] Examples of disorders associated with growth or metabolism of fat tissue include, but are not limited to, rapid weight loss or weight gain, obesity, anorexia, bulimia, diabetes, generalized or familial partial lipodystrophy (peripheral fat wasting), hypercholesterolemia, hyperlipidemia, and other diseases of metabolic rate.

[0048] Thus, the NT69 molecules can act as novel diagnostic targets and therapeutic agents for controlling disorders in, e.g., cell growth and proliferation, neurotransmission, vascular tone, and the proliferation or metabolism of brain cells and fat cells.

[0049] In a preferred embodiment, the pain disorder is inflammatory pain, a neuralgia, nerve entrapment syndromes, or pain associated with a musculoskeletal disorder.

[0050] The NT69 protein, fragments thereof, and derivatives and other variants of the sequence in SEQ ID NO:2 thereof are collectively referred to as "polypeptides or proteins of the invention" or "NT69 polypeptides or proteins." Nucleic acid molecules encoding such polypeptides or proteins are collectively referred to as "nucleic acids of the invention" or “NT69 nucleic acids." NT69 molecules refer to NT69 nucleic acids, polypeptides, and antibodies.

[0051] As used herein, the term "nucleic acid molecule" includes DNA molecules (e.g., a cDNA or genomic DNA) and RNA molecules (e.g., an mRNA) and analogs of the DNA or RNA generated, e.g., by the use of nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

[0052] The term "isolated or purified nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with

which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and/or 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of 5' and/or 3' nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

[0053] As used herein, the term "hybridizes under stringent conditions" describes conditions for hybridization and washing. Stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Aqueous and nonaqueous methods are described in that reference and either can be used. A preferred example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50°C. Another example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 55°C. A further example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C. Preferably, stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C. Particularly preferred stringency conditions (and the conditions that should be used if the practitioner is uncertain about what conditions should be applied to determine if a molecule is within a hybridization limitation of the invention) are hybridization in 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 or 3, corresponds to a naturally-occurring nucleic acid molecule.

[0054] As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein).

[0055] As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding an NT69 protein, preferably a mammalian NT69 protein, and can further include non-coding regulatory sequences, and introns.

[0056] An "isolated" or "purified" polypeptide or protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. In one embodiment, the language "substantially free" means preparation of NT69 protein having less than about 30%, 20%, 10% and more preferably 5% (by dry weight), of non-NT69 protein (also referred to herein as a "contaminating protein"), or of chemical precursors or non-NT69 chemicals. When the NT69 protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation. The invention includes isolated or purified preparations of at least 0.01, 0.1, 1.0, and 10 milligrams in dry weight.

[0057] A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of NT69 (e.g., the sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533) without abolishing or more preferably, without substantially altering a biological activity, whereas an "essential" amino acid residue results in such a change. For example, amino acid residues that are conserved among the polypeptides of the present invention, e.g., those present in the nucleoside transporter domain, are predicted to be particularly unamenable to alteration.

[0058] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine,

leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in an NT69 protein is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of an NT69 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for NT69 biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

[0059] As used herein, a "biologically active portion" of an NT69 protein includes a fragment of an NT69 protein which participates in an interaction between an NT69 molecule and a non-NT69 molecule. Biologically active portions of an NT69 protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the NT69 protein, e.g., the amino acid sequence shown in SEQ ID NO:2, which include less amino acids than the full length NT69 proteins, and exhibit at least one activity of an NT69 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the NT69 protein, e.g., the ability to transport nucleosides or nucleoside analogs across cell membranes. A biologically active portion of an NT69 protein can be a polypeptide which is, for example, 10, 25, 50, 100, 200 or more amino acids in length. Biologically active portions of an NT69 protein can be used as targets for developing agents which modulate an NT69 mediated activity, e.g., nucloside transport.

[0060] Calculations of homology or sequence identity between sequences (the terms are used interchangeably herein) are performed as follows.

[0061] To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence (e.g., when aligning a second sequence to the NT69

amino acid sequence of SEQ ID NO:2 having 475 amino acid residues, at least 190, preferably at least 238, more preferably at least 285, even more preferably at least 333, and even more preferably at least 380, or 428 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0062] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) *J Mol Biol* 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is within a sequence identity or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0063] The percent identity between two amino acid or nucleotide sequences can be determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[0064] The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul et al. (1990) *J. Mol. Biol.* 215:403-10. BLAST

nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to NT69 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to NT69 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

[0065] "Misexpression or aberrant expression", as used herein, refers to a non-wild type pattern of gene expression, at the RNA or protein level. It includes: expression at non-wild type levels, i.e., over or under expression; a pattern of expression that differs from wild type in terms of the time or stage at which the gene is expressed, e.g., increased or decreased expression (as compared with wild type) at a predetermined developmental period or stage; a pattern of expression that differs from wild type in terms of decreased expression (as compared with wild type) in a predetermined cell type or tissue type; a pattern of expression that differs from wild type in terms of the splicing size, amino acid sequence, post-transitional modification, or biological activity of the expressed polypeptide; a pattern of expression that differs from wild type in terms of the effect of an environmental stimulus or extracellular stimulus on expression of the gene, e.g., a pattern of increased or decreased expression (as compared with wild type) in the presence of an increase or decrease in the strength of the stimulus.

[0066] "Subject", as used herein, can refer to a mammal, e.g., a human, or to an experimental or animal or disease model. The subject can also be a non-human animal, e.g., a horse, cow, goat, or other domestic animal.

[0067] A "purified preparation of cells", as used herein, refers to, in the case of plant or animal cells, an in vitro preparation of cells and not an entire intact plant or animal. In the case of cultured cells or microbial cells, it consists of a preparation of at least 10% and more preferably 50% of the subject cells.

[0068] Various aspects of the invention are described in further detail below.

Isolated Nucleic Acid Molecules

[0069] In one aspect, the invention provides, an isolated or purified, nucleic acid molecule that encodes an NT69 polypeptide described herein, e.g., a full length NT69 protein

or a fragment thereof, e.g., a biologically active portion of NT69 protein. Also included is a nucleic acid fragment suitable for use as a hybridization probe, which can be used, e.g., to identify a nucleic acid molecule encoding a polypeptide of the invention, NT69 mRNA, and fragments suitable for use as primers, e.g., PCR primers for the amplification or mutation of nucleic acid molecules.

[0070] In one embodiment, an isolated nucleic acid molecule of the invention includes the nucleotide sequence shown in SEQ ID NO:1, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, or a portion of any of these nucleotide sequences. In one embodiment, the nucleic acid molecule includes sequences encoding the human NT69 protein (i.e., "the coding region", from about nucleotide 52 to about 1477 of SEQ ID NO:1), as well as 5' untranslated sequences (nucleotides 1 to 51 of SEQ ID NO:1). Alternatively, the nucleic acid molecule can include only the coding region of SEQ ID NO:1 (e.g., nucleotides 52 to 1477, corresponding to SEQ ID NO:3) and, e.g., no flanking sequences which normally accompany the subject sequence. In another embodiment, the nucleic acid molecule encodes a sequence corresponding to the mature protein from about amino acid 1 to amino acid 475 of SEQ ID NO:2.

[0071] In another embodiment, an isolated nucleic acid molecule of the invention includes a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number PTA-2533, or a portion of any of these nucleotide sequences. In other embodiments, the nucleic acid molecule of the invention is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533 such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, thereby forming a stable duplex.

[0072] In one embodiment, an isolated nucleic acid molecule of the present invention includes a nucleotide sequence which is at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous to the entire length of the nucleotide sequence shown in SEQ ID NO:1 or 3, or the entire length of the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, or a portion, preferably of the same length, of any of these nucleotide sequences.

NT69 Nucleic Acid Fragments

[0073] A nucleic acid molecule of the invention can include only a portion of the nucleic acid sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533. For example, such a nucleic acid molecule can include a fragment which can be used as a probe or primer or a fragment encoding a portion of an NT69 protein, e.g., an immunogenic or biologically active portion of an NT69 protein. A fragment can comprise nucleotides 345 to 1356 of SEQ ID NO:3, which encodes a nucleoside transporter signature domain of human NT69. The nucleotide sequence determined from the cloning of the NT69 gene allows for the generation of probes and primers designed for use in identifying and/or cloning other NT69 family members, or fragments thereof, as well as NT69 homologues, or fragments thereof, from other species.

[0074] In another embodiment, a nucleic acid includes a nucleotide sequence that includes part, or all, of the coding region and extends into either (or both) the 5' or 3' noncoding region. Other embodiments include a fragment which includes a nucleotide sequence encoding an amino acid fragment described herein. Nucleic acid fragments can encode a specific domain or site described herein or fragments thereof, particulary fragments thereof which are at least 30 amino acids in length. Fragments also include nucleic acid sequences corresponding to specific amino acid sequences described above or fragments thereof. Nucleic acid fragments should not to be construed as encompassing those fragments that may have been disclosed prior to the invention.

[0075] A nucleic acid fragment can include a sequence corresponding to a domain, region, or functional site described herein. A nucleic acid fragment can also include one or more domain, region, or functional site described herein. Thus, for example, a nucleic acid fragment can comprise nucleotides 345 to 1356 of SEQ ID NO:3, which encodes a nucleoside transporter signature domain of human NT69.

[0076] NT69 probes and primers are provided. Typically a probe/primer is an isolated or purified oligonucleotide. The oligonucleotide typically includes a region of nucleotide sequence that hybridizes under stringent conditions to at least about 7, 12 or 15, preferably about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense or antisense sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, or of a naturally occurring allelic variant or mutant of SEQ ID NO:1 or 3, or the

nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533.

[0077] In a preferred embodiment the nucleic acid is a probe which is at least 5 or 10, and less than 200, more preferably less than 100, or less than 50, base pairs in length. It should be identical, or differ by 1, or less than in 5 or 10 bases, from a sequence disclosed herein. If alignment is needed for this comparison the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.

[0078] A probe or primer can be derived from the sense or anti-sense strand of a nucleic acid which encodes amino acid residues 115 to 452 of SEQ ID NO: 2.

[0079] In another embodiment a set of primers is provided, e.g., primers suitable for use in a PCR, which can be used to amplify a selected region of an NT69 sequence, e.g., a domain, region, site or other sequence described herein. The primers should be at least 5, 10, or 50 base pairs in length and less than 100, or less than 200, base pairs in length. The primers should be identical, or differs by one base from a sequence disclosed herein or from a naturally occurring variant. For example, primers suitable for amplifying all or a portion of any of the following regions are provided: amino acid residues 115 to 452 of SEQ ID NO: 2.

[0080] A nucleic acid fragment can encode an epitope bearing region of a polypeptide described herein.

[0081] A nucleic acid fragment encoding a "biologically active portion of an NT69 polypeptide" can be prepared by isolating a portion of the nucleotide sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, which encodes a polypeptide having an NT69 biological activity (e.g., the biological activities of the NT69 proteins are described herein), expressing the encoded portion of the NT69 protein (e.g., by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the NT69 protein. For example, a nucleic acid fragment encoding a biologically active portion of NT69 includes a nucleoside transporter signature domain, e.g., amino acid residues 115 to 452 of SEQ ID NO:2. A nucleic acid fragment encoding a biologically active portion of an NT69 polypeptide, may comprise a nucleotide sequence which is greater than 300 or more nucleotides in length.

[0082] In preferred embodiments, nucleic acids include a nucleotide sequence which is about 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000 nucleotides

in length and hybridizes under stringent hybridization conditions to a nucleic acid molecule of SEQ ID NO:1, or SEQ ID NO:3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533.

NT69 Nucleic Acid Variants

[0083] The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533. Such differences can be due to degeneracy of the genetic code (and result in a nucleic acid which encodes the same NT69 proteins as those encoded by the nucleotide sequence disclosed herein). In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence which differs, by at least 1, but less than 5, 10, 20, 50, or 100 amino acid residues that shown in SEQ ID NO:2. If alignment is needed for this comparison the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.

[0084] Nucleic acids of the invention can be chosen for having codons, which are preferred, or non preferred, for a particular expression system. E.g., the nucleic acid can be one in which at least one codon, and preferably at least 10%, or 20% of the codons has been altered such that the sequence is optimized for expression in bacterial (e.g., *E. coli*), yeast, human, insect, or nonhuman mammalian (e.g., Chinese hamster ovary (CHO)) cells.

[0085] Nucleic acid variants can be naturally occurring, such as allelic variants (same locus), homologs (different locus), and orthologs (different organism) or can be non naturally occurring. Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product).

[0086] In a preferred embodiment, the nucleic acid differs from that of SEQ ID NO: 1 or 3, or the sequence in ATCC® Accession Number 2533, e.g., as follows: by at least one but less than 10, 20, 30, or 40 nucleotides; at least one but less than 1%, 5%, 10% or 20% of the nucleotides in the subject nucleic acid. If necessary for this analysis the sequences should be

aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences.

[0087] Orthologs, homologs, and allelic variants can be identified using methods known in the art. These variants comprise a nucleotide sequence encoding a polypeptide that is 50%, at least about 55%, typically at least about 70-75%, more typically at least about 80-85%, and most typically at least about 90-95% or more identical to the nucleotide sequence shown in SEQ ID NO:2 or a fragment of this sequence. Such nucleic acid molecules can readily be identified as being able to hybridize under stringent conditions, to the nucleotide sequence shown in SEQ ID NO 2 or a fragment of the sequence. Nucleic acid molecules corresponding to orthologs, homologs, and allelic variants of the NT69 cDNAs of the invention can further be isolated by mapping to the same chromosome or locus as the NT69 gene. Preferred variants include those that can catalyze transport of a nucleoside across a cell membrane.

[0088] Allelic variants of NT69, e.g., human NT69, include both functional and non-functional proteins. Functional allelic variants are naturally occurring amino acid sequence variants of the NT69 protein within a population that maintain the ability to transport nucleosides or nucleoside analogs across a cell membrane. Functional allelic variants will typically contain only conservative substitution of one or more amino acids of SEQ ID NO:2, or substitution, deletion or insertion of non-critical residues in non-critical regions of the protein. Non-functional allelic variants are naturally-occurring amino acid sequence variants of the NT69, e.g., human NT69, protein within a population that do not have the ability to catalyze nucleoside transport across a cell membrane. Non-functional allelic variants will typically contain a non-conservative substitution, a deletion, or insertion, or premature truncation of the amino acid sequence of SEQ ID NO:2, or a substitution, insertion, or deletion in critical residues or critical regions of the protein.

[0089] Moreover, nucleic acid molecules encoding other NT69 family members and, thus, which have a nucleotide sequence which differs from the NT69 sequences of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533 are intended to be within the scope of the invention.

Antisense Nucleic Acid Molecules, Ribozymes and Modified NT69 Nucleic Acid Molecules

[0090] In another aspect, the invention features, an isolated nucleic acid molecule which is antisense to NT69. An "antisense" nucleic acid can include a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. The antisense nucleic acid can be complementary to an entire NT69 coding strand, or to only a portion thereof (e.g., the coding region of human NT69 corresponding to SEQ ID NO:3). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding NT69 (e.g., the 5' and 3' untranslated regions).

[0091] An antisense nucleic acid can be designed such that it is complementary to the entire coding region of NT69 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of NT69 mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of NT69 mRNA, e.g., between the -10 and +10 regions of the target gene nucleotide sequence of interest. An antisense oligonucleotide can be, for example, about 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more nucleotides in length.

[0092] An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. The antisense nucleic acid also can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

[0093] The antisense nucleic acid molecules of the invention are typically administered to a subject (e.g., by direct injection at a tissue site), or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding an NT69 protein to thereby inhibit expression of the protein, e.g., by inhibiting transcription and/or translation.

Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

[0094] In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids. Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

[0095] In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. A ribozyme having specificity for an NT69-encoding nucleic acid can include one or more sequences complementary to the nucleotide sequence of an NT69 cDNA disclosed herein (i.e., SEQ ID NO:1 or 3), and a sequence having known catalytic sequence responsible for mRNA cleavage (see U.S. Pat. No. 5,093,246 or Haselhoff and Gerlach (1988) *Nature* 334:585-591). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in an NT69-encoding mRNA. See, e.g., Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742. Alternatively, NT69 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, e.g., Bartel and Szostak (1993) *Science* 261:1411-1418.

[0096] NT69 gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the NT69 (e.g., the NT69 promoter and/or enhancers) to form triple helical structures that prevent transcription of the NT69 gene in target cells. See generally, Helene (1991) *Anticancer Drug Des.* 6(6):569-84; Helene et al. (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14(12):807-15. The potential sequences that can be targeted for triple helix formation can be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are

synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

[0097] The invention also provides detectably labeled oligonucleotide primer and probe molecules. Typically, such labels are chemiluminescent, fluorescent, radioactive, or colorimetric.

[0098] A NT69 nucleic acid molecule can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup et al. (1996) *Bioorganic & Medicinal Chemistry* 4:5-23). As used herein, the terms "peptide nucleic acid" or "PNA" refers to a nucleic acid mimic, e.g., a DNA mimic, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of a PNA can allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al. (1996) *supra*; Perry-O'Keefe et al. *Proc. Natl. Acad. Sci.* 93: 14670-675.

[0099] PNAs of NT69 nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of NT69 nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (e.g., by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (e.g., S1 nucleases (Hyrup et al. (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup et al. (1996) *supra*; Perry-O'Keefe *supra*).

[00100] In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, e.g., PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, e.g., Krol et al. (1988) *Bio-Techniques* 6:958-976) or intercalating agents. (See, e.g., Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may

be conjugated to another molecule, (e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

[00101] The invention also includes molecular beacon oligonucleotide primer and probe molecules having at least one region which is complementary to an NT69 nucleic acid of the invention, two complementary regions one having a fluorophore and one a quencher such that the molecular beacon is useful for quantitating the presence of the NT69 nucleic acid of the invention in a sample. Molecular beacon nucleic acids are described, for example, in Lizardi et al., U.S. Patent No. 5,854,033; Nazarenko et al., U.S. Patent No. 5,866,336, and Livak et al., U.S. Patent 5,876,930.

<u>Isolated NT69 Polypeptides</u>

[00102] In another aspect, the invention features, an isolated NT69 protein, or fragment, e.g., a biologically active portion, for use as immunogens or antigens to raise or test (or more generally to bind) anti-NT69 antibodies. NT69 protein can be isolated from cells or tissue sources using standard protein purification techniques. NT69 protein or fragments thereof can be produced by recombinant DNA techniques or synthesized chemically.

[00103] Polypeptides of the invention include those which arise as a result of the existence of multiple genes, alternative transcription events, alternative RNA splicing events, and alternative translational and postranslational events. The polypeptide can be expressed in systems, e.g., cultured cells, which result in substantially the same postranslational modifications present when expressed the polypeptide is expressed in a native cell, or in systems which result in the alteration or omission of postranslational modifications, e.g., gylcosylation or cleavage, present when expressed in a native cell.

[00104] In a preferred embodiment, An NT69 polypeptide has one or more of the following characteristics:

(i) it is involved in the metabolic changes that occur as a result of excess energy input;

(ii) it has the ability to catalyze the transport of a nucleoside or nucleoside analog across a cell membrane;

(iii) it has a molecular weight, e.g., a deduced molecular weight, preferably ignoring any contribution of post translational modifications, amino acid composition or other physical characteristic of SEQ ID NO:2;

(iv) it has an overall sequence similarity of at least 60%, more preferably at least 70, 80, 90, or 95%, with a polypeptide of SEQ ID NO:2;

(v) it can be found in high levels in brain, liver, and fat tissue;

(vi) it has a nucleoside transporter signature domain which is preferably about 70%, 80%, 90% or 95% identical to amino acid residues 115 to 452 of SEQ ID NO:2; and

(vii) it has at least 5, preferably 8, and most preferably 11 of the cysteines found amino acid sequence of the native protein.

[00105] In a preferred embodiment the NT69 protein, or fragment thereof, differs from the corresponding sequence in SEQ ID NO:2. In one embodiment it differs by at least one but by less than 15, 10 or 5 amino acid residues. In another it differs from the corresponding sequence in SEQ ID NO:2 by at least one residue but less than 20%, 15%, 10% or 5% of the residues in it differ from the corresponding sequence in SEQ ID NO:2. If this comparison requires alignment the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences. The differences are, preferably, differences or changes at a non-essential residue or a conservative substitution. In a preferred embodiment the differences are not in amino acids 115 to 452. In another preferred embodiment one or more differences are in 1 to 114, or 453 to 475.

[00106] Useful NT69 polypeptides include those comprising 15, 30, 40, 50, 60, 75, 100, 125, 150, 300, 350, 400, 425, 450, 460, or 470 contiguous amino acids of SEQ ID NO:2.

[00107] Other embodiments include a protein that contain one or more changes in amino acid sequence, e.g., a change in an amino acid residue which is not essential for activity. Such NT69 proteins differ in amino acid sequence from SEQ ID NO:2, yet retain biological activity.

[00108] In one embodiment, the protein includes an amino acid sequence at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or more homologous to SEQ ID NO:2.

[00109] An NT69 protein or fragment is provided which varies from the sequence of SEQ ID NO:2 in regions of amino acids 1 to 114, and from 453 to 475 by at least one but by less than 15, 10 or 5 amino acid residues in the protein or fragment but which does not differ from SEQ ID NO:2 in regions 115 to 452. If this comparison requires alignment the sequences should be aligned for maximum homology. "Looped" out sequences from deletions or insertions, or mismatches, are considered differences. In some embodiments the difference is at a non-essential residue or is a conservative substitution, while in others the difference is at an essential residue or is a non conservative substitution.

[00110] In one embodiment, a biologically active portion of an NT69 protein includes a nucleoside transporter signature domain. Moreover, other biologically active portions, in

which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native NT69 protein.

[00111] In a preferred embodiment, the NT69 protein has an amino acid sequence shown in SEQ ID NO:2. In other embodiments, the NT69 protein is substantially identical to SEQ ID NO:2. In yet another embodiment, the NT69 protein is substantially identical to SEQ ID NO:2 and retains the functional activity of the protein of SEQ ID NO:2, as described in detail in the subsections above.

NT69 Chimeric or Fusion Proteins

[00112] In another aspect, the invention provides NT69 chimeric or fusion proteins. As used herein, an NT69 "chimeric protein" or "fusion protein" includes an NT69 polypeptide linked to a non-NT69 polypeptide. A "non-NT69 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the NT69 protein, e.g., a protein which is different from the NT69 protein and which is derived from the same or a different organism. The NT69 polypeptide of the fusion protein can correspond to all or a portion e.g., a fragment described herein of an NT69 amino acid sequence. In a preferred embodiment, an NT69 fusion protein includes at least one biologically active portion of an NT69 protein. The non-NT69 polypeptide can be fused to the N-terminus or C-terminus of the NT69 polypeptide.

[00113] The fusion protein can include a moiety which has a high affinity for a ligand. For example, the fusion protein can be a GST-NT69 fusion protein in which the NT69 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant NT69. Alternatively, the fusion protein can be an NT69 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (e.g., mammalian host cells), expression and/or secretion of NT69 can be increased through use of a heterologous signal sequence.

[00114] Fusion proteins can include all or a part of a serum protein, e.g., an IgG constant region, or human serum albumin.

[00115] The NT69 fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The NT69 fusion proteins can be used to affect the bioavailability of an NT69 substrate. NT69 fusion proteins may be useful therapeutically for the treatment of disorders caused by, for example, (i)

aberrant modification or mutation of a gene encoding an NT69 protein; (ii) mis-regulation of the NT69 gene; and (iii) aberrant post-translational modification of an NT69 protein.

[00116] Moreover, the NT69-fusion proteins of the invention can be used as immunogens to produce anti-NT69 antibodies in a subject, to purify NT69 ligands and in screening assays to identify molecules that inhibit the interaction of NT69 with an NT69 substrate, e.g., a nucleoside.

[00117] Expression vectors are commercially available that already encode a fusion moiety (e.g., a GST polypeptide). An NT69-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the NT69 protein.

<u>Variants of NT69 Proteins</u>

[00118] In another aspect, the invention also features a variant of an NT69 polypeptide, e.g., which functions as an agonist (mimetics) or as an antagonist. Variants of the NT69 proteins can be generated by mutagenesis, e.g., discrete point mutation, the insertion or deletion of sequences or the truncation of an NT69 protein. An agonist of the NT69 proteins can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of an NT69 protein. An antagonist of an NT69 protein can inhibit one or more of the activities of the naturally occurring form of the NT69 protein by, for example, competitively modulating an NT69-mediated activity of an NT69 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function. Preferably, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the NT69 protein.

[00119] Variants of an NT69 protein can be identified by screening combinatorial libraries of mutants, e.g., truncation mutants, of an NT69 protein for agonist or antagonist activity.

[00120] Libraries of fragments e.g., N terminal, C terminal, or internal fragments, of an NT69 protein coding sequence can be used to generate a variegated population of fragments for screening and subsequent selection of variants of an NT69 protein.

[00121] Variants in which a cysteine residue is added or deleted or in which a residue which is glycosylated is added or deleted are particularly preferred.

[00122] Methods for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property are known. Recursive ensemble mutagenesis (REM), a new technique which

enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify NT69 variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave et al. (1993) *Protein Engineering* 6(3):327-331).

[00123] Cell based assays can be exploited to analyze a variegated NT69 library. For example, a library of expression vectors can be transfected into a cell line, e.g., a cell line, which ordinarily responds to NT69 substrates in a substrate-dependent manner. The transfected cells are then contacted with NT69 substrates and the effect of the expression of the mutant on signaling by the NT69 substrate can be detected, e.g., by measuring transport of nucleosides across a plasma membrane or binding by NBMPR. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of signaling by the NT69 substrate, and the individual clones further characterized.

[00124] In another aspect, the invention features a method of making an NT69 polypeptide, e.g., a peptide having a non-wild type activity, e.g., an antagonist, agonist, or super agonist of a naturally occurring NT69 polypeptide, e.g., a naturally occurring NT69 polypeptide. The method includes: altering the sequence of an NT69 polypeptide, e.g., altering the sequence, e.g., by substitution or deletion of one or more residues of a non-conserved region, a domain or residue disclosed herein, and testing the altered polypeptide for the desired activity.

[00125] In another aspect, the invention features a method of making a fragment or analog of an NT69 polypeptide a biological activity of a naturally occurring NT69 polypeptide. The method includes: altering the sequence, e.g., by substitution or deletion of one or more residues, of an NT69 polypeptide, e.g., altering the sequence of a non-conserved region, or a domain or residue described herein, and testing the altered polypeptide for the desired activity.

<u>Anti-NT69 Antibodies</u>

[00126] In another aspect, the invention provides an anti-NT69 antibody. The term "antibody" as used herein refers to an immunoglobulin molecule or immunologically active portion thereof, i.e., an antigen-binding portion. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin.

[00127] The antibody can be a polyclonal, monoclonal, recombinant, e.g., a chimeric or humanized, fully human, non-human, e.g., murine, or single chain antibody. In a preferred

embodiment it has effector function and can fix complement. The antibody can be coupled to a toxin or imaging agent.

[00128] A full-length NT69 protein or, antigenic peptide fragment of NT69 can be used as an immunogen or can be used to identify anti-NT69 antibodies made with other immunogens, e.g., cells, membrane preparations, and the like. The antigenic peptide of NT69 should include at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an epitope of NT69. Preferably, the antigenic peptide includes at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

[00129] Fragments of NT69 that include residues 205 to 225 can be used to make, e.g., used as immunogens or used to characterize the specificity of an antibody, antibodies against hydrophilic regions of the NT69 protein. Similarly, a fragment of NT69 which includes residues 50 to 70, or 360 to 380 can be used to make an antibody against a hydrophobic region of the NT69 protein; a fragment of NT69 which include residues 115 to 452 can be used to make an antibody against the nucleoside transporter signature region of the NT69 protein.

[00130] Antibodies reactive with, or specific for, any of these regions, or other regions or domains described herein are provided.

[00131] Preferred epitopes encompassed by the antigenic peptide are regions of NT69 are located on the surface of the protein, e.g., hydrophilic regions, as well as regions with high antigenicity. For example, an Emini surface probability analysis of the human NT69 protein sequence can be used to indicate the regions that have a particularly high probability of being localized to the surface of the NT69 protein and are thus likely to constitute surface residues useful for targeting antibody production.

[00132] In a preferred embodiment the antibody binds an epitope on any domain or region on NT69 proteins described herein.

[00133] Chimeric, humanized, but most preferably, completely human antibodies are desirable for applications which include repeated administration, e.g., therapeutic treatment (and some diagnostic applications) of human patients.

[00134] The anti-NT69 antibody can be a single chain antibody. A single-chain antibody (scFV) may be engineered (see, for example, Colcher, D et al. *Ann N Y Acad Sci* (1999) 880:263-80; and Reiter, Y *Clin Cancer Res* (1996) 2:245-52). The single chain antibody can

be dimerized or multimerized to generate multivalent antibodies having specificities for different epitopes of the same target NT69 protein.

[00135] In a preferred embodiment, the antibody has reduced or no ability to bind an Fc receptor. E.g., it is a isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor, e.g., it has a mutagenized or deleted Fc receptor binding region.

[00136] An anti-NT69 antibody (e.g., monoclonal antibody) can be used to isolate NT69 by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, an anti-NT69 antibody can be used to detect NT69 protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the protein. Anti-NT69 antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance (i.e., antibody labeling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

<u>Recombinant Expression Vectors, Host Cells and Genetically Engineered Cells</u>

[00137] In another aspect, the invention includes, vectors, preferably expression vectors, containing a nucleic acid encoding a polypeptide described herein. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked and can include a plasmid, cosmid or viral vector. The vector can be capable of autonomous replication or it can integrate into a host DNA. Viral vectors include, e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses.

[00138] A vector can include an NT69 nucleic acid in a form suitable for expression of the nucleic acid in a host cell. Preferably the recombinant expression vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed.

The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or polypeptides, including fusion proteins or polypeptides, encoded by nucleic acids as described herein (e.g., NT69 proteins, mutant forms of NT69 proteins, fusion proteins, and the like).

[00139] The recombinant expression vectors of the invention can be designed for expression of NT69 proteins in prokaryotic or eukaryotic cells. For example, polypeptides of the invention can be expressed in bacterial cells (e.g., *E. coli*), insect cells (e.g., using baculovirus expression vectors), yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

[00140] Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

[00141] Purified fusion proteins can be used in NT69 activity assays, (e.g., direct assays or competitive assays described in detail below), or to generate antibodies specific for NT69 proteins. In a preferred embodiment, a fusion protein expressed in a retroviral expression vector of the present invention can be used to infect bone marrow cells which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (e.g., six (6) weeks).

[00142] To maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

[00143] The NT69 expression vector can be a yeast expression vector, a vector for expression in insect cells, e.g., a baculovirus expression vector or a vector suitable for expression in mammalian cells.

[00144] When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

[00145] In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert et al. (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji et al. (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund et al. (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example, the murine hox promoters (Kessel and Gruss (1990)

Science 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

[00146] The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. Regulatory sequences (e.g., viral promoters and/or enhancers) operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the constitutive, tissue specific or cell type specific expression of antisense RNA in a variety of cell types. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

[00147] Another aspect the invention provides a host cell which includes a nucleic acid molecule described herein, e.g., an NT69 nucleic acid molecule within a recombinant expression vector or an NT69 nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[00148] A host cell can be any prokaryotic or eukaryotic cell. For example, an NT69 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as CHO or SV40-transformed African green monkey kidney (COS) cells). Other suitable host cells are known to those skilled in the art.

[00149] Vector DNA can be introduced into host cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation

[00150] A host cell of the invention can be used to produce (i.e., express) an NT69 protein. Accordingly, the invention further provides methods for producing an NT69 protein using the host cells of the invention. In one embodiment, the method includes culturing the host cell of the invention (into which a recombinant expression vector encoding an NT69 protein has

been introduced) in a suitable medium such that an NT69 protein is produced. In another embodiment, the method further includes isolating an NT69 protein from the medium or the host cell.

[00151] In another aspect, the invention features, a cell or purified preparation of cells which include an NT69 transgene, or which otherwise misexpress NT69. The cell preparation can consist of human or non human cells, e.g., rodent cells, e.g., mouse or rat cells, rabbit cells, or pig cells. In preferred embodiments, the cell or cells include an NT69 transgene, e.g., a heterologous form of an NT69, e.g., a gene derived from humans (in the case of a non-human cell). The NT69 transgene can be misexpressed, e.g., overexpressed or underexpressed. In other preferred embodiments, the cell or cells include a gene which misexpress an endogenous NT69, e.g., a gene the expression of which is disrupted, e.g., a knockout. Such cells can serve as a model for studying disorders which are related to mutated or mis-expressed NT69 alleles or for use in drug screening.

[00152] In another aspect, the invention features, a human cell, e.g., a hematopoietic stem cell, transformed with nucleic acid which encodes a subject NT69 polypeptide.

[00153] Also provided are cells, preferably human cells, e.g., human hematopoietic or fibroblast cells, in which an endogenous NT69 is under the control of a regulatory sequence that does not normally control the expression of the endogenous NT69 gene. The expression characteristics of an endogenous gene within a cell, e.g., a cell line or microorganism, can be modified by inserting a heterologous DNA regulatory element into the genome of the cell such that the inserted regulatory element is operably linked to the endogenous NT69 gene. For example, an endogenous NT69 gene which is "transcriptionally silent," e.g., not normally expressed, or expressed only at very low levels, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed gene product in that cell. Techniques such as targeted homologous recombinations, can be used to insert the heterologous DNA as described in, e.g., Chappel, US 5,272,071; WO 91/06667, published in May 16, 1991.

Transgenic Animals

[00154] The invention provides non-human transgenic animals. Such animals are useful for studying the function and/or activity of an NT69 protein and for identifying and/or evaluating modulators of NT69 activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in

which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA or a rearrangment, e.g., a deletion of endogenous chromosomal DNA, which preferably is integrated into or occurs in the genome of the cells of a transgenic animal. A transgene can direct the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal, other transgenes, e.g., a knockout, reduce expression. Thus, a transgenic animal can be one in which an endogenous NT69 gene has been altered by, e.g., by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

[00155] Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a transgene of the invention to direct expression of an NT69 protein to particular cells. A transgenic founder animal can be identified based upon the presence of an NT69 transgene in its genome and/or expression of NT69 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding an NT69 protein can further be bred to other transgenic animals carrying other transgenes.

[00156] NT69 proteins or polypeptides can be expressed in transgenic animals or plants, e.g., a nucleic acid encoding the protein or polypeptide can be introduced into the genome of an animal. In preferred embodiments the nucleic acid is placed under the control of a tissue specific promoter, e.g., a milk or egg specific promoter, and recovered from the milk or eggs produced by the animal. Suitable animals are mice, pigs, cows, goats, and sheep.

[00157] The invention also includes a population of cells from a transgenic animal, as discussed, e.g., below.

Uses

[00158] The nucleic acid molecules, proteins, protein homologues, and antibodies described herein can be used in one or more of the following methods: a) screening assays; b) predictive medicine (e.g., diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics); and c) methods of treatment (e.g., therapeutic and prophylactic). The isolated nucleic acid molecules of the invention can be used, for example, to express an NT69

protein (e.g., via a recombinant expression vector in a host cell in gene therapy applications), to detect an NT69 mRNA (e.g., in a biological sample) or a genetic alteration in an NT69 gene, and to modulate NT69 activity, as described further below. The NT69 proteins can be used to treat disorders characterized by insufficient or excessive production of an NT69 substrate or production of NT69 inhibitors. In addition, the NT69 proteins can be used to screen for naturally occurring NT69 substrates, to screen for drugs or compounds which modulate NT69 activity, as well as to treat disorders characterized by insufficient or excessive production of NT69 protein or production of NT69 protein forms which have decreased, aberrant or unwanted activity compared to NT69 wild type protein (e.g., faulty nucleoside binding or transport activity). Moreover, the anti-NT69 antibodies of the invention can be used to detect and isolate NT69 proteins, regulate the bioavailability of NT69 proteins, and modulate NT69 activity.

[00159] A method of evaluating a compound for the ability to interact with, e.g., bind, a subject NT69 polypeptide is provided. The method includes: contacting the compound with the NT69 polypeptide; and evaluating ability of the compound to interact with, e.g., to bind or form a complex with the NT69 polypeptide. This method can be performed in vitro, e.g., in a cell free system, or in vivo, e.g., in a two-hybrid interaction trap assay. This method can be used to identify naturally occurring molecules which interact with NT69 polypeptide. It can also be used to find natural or synthetic inhibitors of NT69 polypeptide. Screening methods are discussed in more detail below.

<u>Screening Assays</u>:

[00160] The invention provides methods (also referred to herein as "screening assays") for identifying modulators, i.e., candidate or test compounds or agents (e.g., proteins, peptides, peptidomimetics, peptoids, small molecules or other drugs) which bind to NT69 proteins, have a stimulatory or inhibitory effect on, for example, NT69 expression or NT69 activity, or have a stimulatory or inhibitory effect on, for example, the expression or activity of an NT69 substrate. Compounds thus identified can be used to modulate the activity of target gene products (e.g., NT69 genes) in a therapeutic protocol, to elaborate the biological function of the target gene product, or to identify compounds that disrupt normal target gene interactions.

[00161] In one embodiment, the invention provides assays for screening candidate or test compounds which are substrates of an NT69 protein or polypeptide or a biologically active portion thereof. In another embodiment, the invention provides assays for screening

candidate or test compounds which bind to or modulate the activity of an NT69 protein or polypeptide or a biologically active portion thereof.

[00162] The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckermann et al. (1994) *J. Med. Chem.* 37: 2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

[00163] Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop et al. (1994) *J. Med. Chem.* 37:1233.

[00164] Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); Devlin (1990) *Science* 249:404-406; Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner, *supra.*).

[00165] In one embodiment, an assay is a cell-based assay in which a cell which expresses an NT69 protein or biologically active portion thereof is contacted with a test compound, and the ability of the test compound to modulate NT69 activity is determined. Determining the ability of the test compound to modulate NT69 activity can be accomplished by monitoring, for example, ability to transport nucleosides across cell membranes (e.g., plasma membranes or organelle membranes). The cell, for example, can be of mammalian origin, e.g., mouse, monkey or human.

[00166] The ability of the test compound to modulate NT69 binding to a compound, e.g., an NT69 substrate, or to bind to NT69 can also be evaluated. This can be accomplished, for example, by coupling the compound, e.g., the substrate, with a radioisotope or enzymatic label such that binding of the compound, e.g., the substrate, to NT69 can be determined by detecting the labeled compound, e.g., substrate, in a complex. Alternatively, NT69 could be coupled with a radioisotope or enzymatic label to monitor the ability of a test compound to modulate NT69 binding to an NT69 substrate in a complex. For example, compounds (e.g., NT69 substrates) can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

[00167] The ability of a compound (e.g., an NT69 substrate) to interact with NT69 with or without the labeling of any of the interactants can be evaluated. For example, a microphysiometer can be used to detect the interaction of a compound with NT69 without the labeling of either the compound or the NT69. McConnell, H. M. et al. (1992) *Science* 257:1906-1912. As used herein, a "microphysiometer" (e.g., Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and NT69.

[00168] In yet another embodiment, a cell-free assay is provided in which an NT69 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the NT69 protein or biologically active portion thereof is evaluated. Preferred biologically active portions of the NT69 proteins to be used in assays of the present invention include fragments that participate in interactions with non-NT69 molecules, e.g., fragments with high surface probability scores.

[00169] Soluble and/or membrane-bound forms of isolated proteins (e.g., NT69 proteins or biologically active portions thereof) can be used in the cell-free assays of the invention. When membrane-bound forms of the protein are used, it may be desirable to utilize a solubilizing agent. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane

sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

[00170] Cell-free assays involve preparing a reaction mixture of the target gene protein and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected.

[00171] The interaction between two molecules can also be detected, e.g., using fluorescence energy transfer (FET) (see, for example, Lakowicz et al., U.S. Patent No. 5,631,169; Stavrianopoulos et al., U.S. Patent No. 4,868,103). A fluorophore label on the first, 'donor' molecule is selected such that its emitted fluorescent energy will be absorbed by a fluorescent label on a second, 'acceptor' molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately, the 'donor' protein molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light, such that the 'acceptor' molecule label may be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter).

[00172] In another embodiment, determining the ability of the NT69 protein to bind to a target molecule can be accomplished using real-time Biomolecular Interaction Analysis (BIA) (see, e.g., Sjolander and Urbaniczky (1991) *Anal. Chem.* 63:2338-2345 and Szabo et al. (1995) *Curr. Opin. Struct. Biol.* 5:699-705). "Surface plasmon resonance" or "BIA" detects biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)), resulting in a detectable signal which can be used as an indication of real-time reactions between biological molecules.

[00173] In one embodiment, the target gene product or the test substance is anchored onto a solid phase. The target gene product/test compound complexes anchored on the solid phase can be detected at the end of the reaction. Preferably, the target gene product can be

anchored onto a solid surface, and the test compound, (which is not anchored), can be labeled, either directly or indirectly, with detectable labels discussed herein.

[00174] It may be desirable to immobilize either NT69, an anti NT69 antibody or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to an NT69 protein, or interaction of an NT69 protein with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/NT69 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or NT69 protein, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of NT69 binding or activity determined using standard techniques.

[00175] Other techniques for immobilizing either an NT69 protein or a target molecule on matrices include using conjugation of biotin and streptavidin. Biotinylated NT69 protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).

[00176] In order to conduct the assay, the non-immobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface;

e.g., using a labeled antibody specific for the immobilized component (the antibody, in turn, can be directly labeled or indirectly labeled with, e.g., a labeled anti-Ig antibody).

[00177] In one embodiment, this assay is performed utilizing antibodies reactive with NT69 protein or target molecules but which do not interfere with binding of the NT69 protein to its target molecule. Such antibodies can be derivatized to the wells of the plate, and unbound target or NT69 protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the NT69 protein or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the NT69 protein or target molecule.

[00178] Alternatively, cell free assays can be conducted in a liquid phase. In such an assay, the reaction products are separated from unreacted components, by any of a number of standard techniques, including but not limited to: differential centrifugation (see, for example, Rivas and Minton (1993) *Trends Biochem Sci* 18(8):284-7); chromatography (gel filtration chromatography, ion-exchange chromatography); electrophoresis (see, e.g., Ausubel et al., eds. Current Protocols in Molecular Biology 1999, J. Wiley: New York.); and immunoprecipitation (see, for example, Ausubel et al., eds. Current Protocols in Molecular Biology 1999, J. Wiley: New York). Such resins and chromatographic techniques are known to one skilled in the art (see, e.g., Heegaard (1998) *J Mol Recognit* 11:141-8; Hage and Tweed (1997) *J Chromatogr. B. Biomed. Sci. Appl.* 699:499-525). Further, fluorescence energy transfer may also be conveniently utilized, as described herein, to detect binding without further purification of the complex from solution.

[00179] In a preferred embodiment, the assay includes contacting the NT69 protein or biologically active portion thereof with a known compound which binds NT69 to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with an NT69 protein, wherein determining the ability of the test compound to interact with an NT69 protein includes determining the ability of the test compound to preferentially bind to NT69 or biologically active portion thereof, or to modulate the activity of a target molecule, as compared to the known compound.

[00180] The target gene products of the invention can, *in vivo*, interact with one or more cellular or extracellular macromolecules, such as proteins. For the purposes of this discussion, such cellular and extracellular macromolecules are referred to herein as "binding partners." Compounds that disrupt such interactions can be useful in regulating the activity

of the target gene product. Such compounds can include, but are not limited to molecules such as antibodies, peptides, and small molecules. The preferred target genes/products for use in this embodiment are the NT69 genes herein identified. In an alternative embodiment, the invention provides methods for determining the ability of the test compound to modulate the activity of an NT69 protein through modulation of the activity of a downstream effector of an NT69 target molecule. For example, the activity of the effector molecule on an appropriate target can be determined, or the binding of the effector to an appropriate target can be determined, as previously described.

[00181] To identify compounds that interfere with the interaction between the target gene product and its cellular or extracellular binding partner(s), a reaction mixture containing the target gene product and the binding partner is prepared, under conditions and for a time sufficient, to allow the two products to form complex. In order to test an inhibitory agent, the reaction mixture is provided in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of the target gene and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target gene product and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the target gene product and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal target gene product can also be compared to complex formation within reaction mixtures containing the test compound and mutant target gene product. This comparison can be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target gene products.

[00182] These assays can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the target gene product or the binding partner onto a solid phase, and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target gene products and the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance.

Alternatively, test compounds that disrupt preformed complexes, e.g., compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are briefly described below.

[00183] In a heterogeneous assay system, either the target gene product or the interactive cellular or extracellular binding partner, is anchored onto a solid surface (e.g., a microtiter plate), while the non-anchored species is labeled, either directly or indirectly. The anchored species can be immobilized by non-covalent or covalent attachments. Alternatively, an immobilized antibody specific for the species to be anchored can be used to anchor the species to the solid surface.

[00184] In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, can be directly labeled or indirectly labeled with, e.g., a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds that inhibit complex formation or that disrupt preformed complexes can be detected.

[00185] Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds that inhibit complex or that disrupt preformed complexes can be identified.

[00186] In an alternate embodiment of the invention, a homogeneous assay can be used. For example, a preformed complex of the target gene product and the interactive cellular or extracellular binding partner product is prepared in that either the target gene products or their binding partners are labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 that utilizes this approach for

immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt target gene product-binding partner interaction can be identified.

[00187] In yet another aspect, the NT69 proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al. (1993) *Cell* 72:223-232; Madura et al. (1993) *J. Biol. Chem.* 268:12046-12054; Bartel et al. (1993) *Biotechniques* 14:920-924; Iwabuchi et al. (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with NT69 ("NT69-binding proteins" or "NT69-bp") and are involved in NT69 activity. Such NT69-bps can be activators or inhibitors of signals by the NT69 proteins or NT69 targets as, for example, downstream elements of an NT69-mediated signaling pathway.

[00188] The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for an NT69 protein is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. (Alternatively the: NT69 protein can be the fused to the activator domain.) If the "bait" and the "prey" proteins are able to interact, *in vivo,* forming an NT69-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the NT69 protein.

[00189] In another embodiment, modulators of NT69 expression are identified. For example, a cell or cell free mixture is contacted with a candidate compound and the expression of NT69 mRNA or protein evaluated relative to the level of expression of NT69 mRNA or protein in the absence of the candidate compound. When expression of NT69 mRNA or protein is greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of NT69 mRNA or protein expression. Alternatively, when expression of NT69 mRNA or protein is less (statistically significantly

less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of NT69 mRNA or protein expression. The level of NT69 mRNA or protein expression can be determined by methods described herein for detecting NT69 mRNA or protein.

[00190] In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell free assay, and the ability of the agent to modulate the activity of an NT69 protein can be confirmed *in vivo*.

[00191] This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein (e.g., an NT69 modulating agent, an antisense NT69 nucleic acid molecule, an NT69-specific antibody, or an NT69-binding partner) in an appropriate animal model to determine the efficacy, toxicity, side effects, or mechanism of action, of treatment with such an agent. Furthermore, novel agents identified by the above-described screening assays can be used for treatments as described herein.

Detection Assays

[00192] Portions or fragments of the nucleic acid sequences identified herein can be used as polynucleotide reagents. For example, these sequences can be used to: (i) map their respective genes on a chromosome e.g., to locate gene regions associated with genetic disease or to associate NT69 with a disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

Chromosome Mapping

[00193] The NT69 nucleotide sequences or portions thereof can be used to map the location of the NT69 genes on a chromosome. This process is called chromosome mapping. Chromosome mapping is useful in correlating the NT69 sequences with genes associated with disease.

[00194] Briefly, NT69 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the NT69 nucleotide sequences. These primers can then be used for PCR screening of somatic cell hybrids containing individual human

chromosomes. Only those hybrids containing the human gene corresponding to the NT69 sequences will yield an amplified fragment.

[00195] A panel of somatic cell hybrids in which each cell line contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, can allow easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. et al. (1983) *Science* 220:919-924).

[00196] Other mapping strategies, e.g., *in situ* hybridization (described in Fan, Y. et al. (1990) *Proc. Natl. Acad. Sci. USA*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries can be used to map NT69 to a chromosomal location.

[00197] Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma et al., Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

[00198] Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

[00199] Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. et al. (1987) *Nature,* 325:783-787.

[00200] Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the NT69 gene, can be determined. If a mutation is

observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

Tissue Typing

[00201] NT69 sequences can be used to identify individuals from biological samples using, e.g., restriction fragment length polymorphism (RFLP). In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, the fragments separated, e.g., in a Southern blot, and probed to yield bands for identification. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

[00202] Furthermore, the sequences of the present invention can also be used to determine the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the NT69 nucleotide sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it. Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences.

[00203] Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1 can provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3 are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

[00204] If a panel of reagents from NT69 nucleotide sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be

used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

Use of Partial NT69 Sequences in Forensic Biology

[00205] DNA-based identification techniques can also be used in forensic biology. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, e.g., hair or skin, or body fluids, e.g., blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

[00206] The sequences of the present invention can be used to provide polynucleotide reagents, e.g., PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (i.e. another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 (e.g., fragments derived from the noncoding regions of SEQ ID NO:1 having a length of at least 20 bases, preferably at least 30 bases) are particularly appropriate for this use.

[00207] The NT69 nucleotide sequences described herein can further be used to provide polynucleotide reagents, e.g., labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such NT69 probes can be used to identify tissue by species and/or by organ type.

[00208] In a similar fashion, these reagents, e.g., NT69 primers or probes can be used to screen tissue culture for contamination (i.e. screen for the presence of a mixture of different types of cells in a culture).

Predictive Medicine

[00209] The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual.

[00210] Generally, the invention provides, a method of determining if a subject is at risk for a disorder related to a lesion in or the misexpression of a gene which encodes NT69. Such disorders include, but are not limited to, e.g., a disorder associated with the misexpression of NT69; a disorder of fat tissue metabolism or proliferation; and a disorder of pain.

[00211] The method includes one or more of the following:

detecting, in a tissue of the subject, the presence or absence of a mutation which affects the expression of the NT69 gene, or detecting the presence or absence of a mutation in a region which controls the expression of the gene, e.g., a mutation in the 5' control region;

detecting, in a tissue of the subject, the presence or absence of a mutation which alters the structure of the NT69 gene;

detecting, in a tissue of the subject, the misexpression of the NT69 gene, at the mRNA level, e.g., detecting a non-wild type level of a mRNA; or

detecting, in a tissue of the subject, the misexpression of the gene, at the protein level, e.g., detecting a non-wild type level of an NT69 polypeptide.

[00212] In preferred embodiments the method includes: ascertaining the existence of at least one of: a deletion of one or more nucleotides from the NT69 gene; an insertion of one or more nucleotides into the gene, a point mutation, e.g., a substitution of one or more nucleotides of the gene, a gross chromosomal rearrangement of the gene, e.g., a translocation, inversion, or deletion.

[00213] For example, detecting the genetic lesion can include: (i) providing a probe/primer including an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence from SEQ ID NO: 3 or naturally occurring mutants thereof or 5' or 3' flanking sequences naturally associated with the NT69 gene; (ii) exposing the probe/primer to nucleic acid of the tissue; and detecting, by hybridization, e.g., *in situ* hybridization, of the probe/primer to the nucleic acid, the presence or absence of the genetic lesion.

[00214] In preferred embodiments detecting the misexpression includes ascertaining the existence of at least one of: an alteration in the level of a messenger RNA transcript of the NT69 gene; the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene; or a non-wild type level of NT69.

[00215] Methods of the invention can be used prenatally or to determine if a subject's offspring will be at risk for a disorder.

[00216] In preferred embodiments the method includes determining the structure of an NT69 gene, an abnormal structure being indicative of risk for the disorder.

[00217] In preferred embodiments the method includes contacting a sample from the subject with an antibody to the NT69 protein or a nucleic acid, which hybridizes specifically with the gene. These and other embodiments are discussed below.

Diagnostic and Prognostic Assays

[00218] The presence, level, or absence of NT69 protein or nucleic acid in a biological sample can be evaluated by obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting NT69 protein or nucleic acid (e.g., mRNA, genomic DNA) that encodes NT69 protein such that the presence of NT69 protein or nucleic acid is detected in the biological sample. The term "biological sample" includes tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. A preferred biological sample is serum. The level of expression of the NT69 gene can be measured in a number of ways, including, but not limited to: measuring the mRNA encoded by the NT69 genes; measuring the amount of protein encoded by the NT69 genes; or measuring the activity of the protein encoded by the NT69 genes.

[00219] The level of mRNA corresponding to the NT69 gene in a cell can be determined both by *in situ* and by *in vitro* formats.

[00220] The isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays. One preferred diagnostic method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe can be, for example, a full-length NT69 nucleic acid, such as the nucleic acid of SEQ ID NO:1, or the DNA insert of the plasmid deposited with ATCC® as Accession Number PTA-2533, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to NT69 mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays are described herein.

[00221] In one format, mRNA (or cDNA) is immobilized on a surface and contacted with the probes, for example by running the isolated mRNA on an agarose gel and transferring the

mRNA from the gel to a membrane, such as nitrocellulose. In an alternative format, the probes are immobilized on a surface and the mRNA (or cDNA) is contacted with the probes, for example, in a two-dimensional gene chip array. A skilled artisan can adapt known mRNA detection methods for use in detecting the level of mRNA encoded by the NT69 genes.

[00222] The level of mRNA in a sample that is encoded by one of NT69 can be evaluated with nucleic acid amplification, e.g., by rtPCR (Mullis (1987) U.S. Patent No. 4,683,202), ligase chain reaction (Barany (1991) *Proc. Natl. Acad. Sci. USA 88*:189-193), self sustained sequence replication (Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA 87*:1874-1878), transcriptional amplification system (Kwoh et al. (1989) *Proc. Natl. Acad. Sci. USA 86*:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) *Biotechnology 6*:1197), rolling circle replication (Lizardi et al., U.S. Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques known in the art. As used herein, amplification primers are defined as being a pair of nucleic acid molecules that can anneal to 5' or 3' regions of a gene (plus and minus strands, respectively, or vice-versa) and contain a short region in between. In general, amplification primers are from about 10 to 30 nucleotides in length and flank a region from about 50 to 200 nucleotides in length. Under appropriate conditions and with appropriate reagents, such primers permit the amplification of a nucleic acid molecule comprising the nucleotide sequence flanked by the primers.

[00223] For *in situ* methods, a cell or tissue sample can be prepared/processed and immobilized on a support, typically a glass slide, and then contacted with a probe that can hybridize to mRNA that encodes the NT69 gene being analyzed.

[00224] In another embodiment, the methods further include contacting a control sample with a compound or agent capable of detecting NT69 mRNA, or genomic DNA, and comparing the presence of NT69 mRNA or genomic DNA in the control sample with the presence of NT69 mRNA or genomic DNA in the test sample.

[00225] A variety of methods can be used to determine the level of protein encoded by NT69. In general, these methods include contacting an agent that selectively binds to the protein, such as an antibody with a sample, to evaluate the level of protein in the sample. In a preferred embodiment, the antibody bears a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used. The term "labeled", with regard to the probe or antibody, is intended to

encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with a detectable substance. Examples of detectable substances are provided herein.

[00226] The detection methods can be used to detect NT69 protein in a biological sample *in vitro* as well as *in vivo*. *In vitro* techniques for detection of NT69 protein include enzyme linked immunosorbent assays (ELISAs), immunoprecipitations, immunofluorescence, enzyme immunoassay (EIA), radioimmunoassay (RIA), and Western blot analysis. *In vivo* techniques for detection of NT69 protein include introducing into a subject a labeled anti-NT69 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

[00227] In another embodiment, the methods further include contacting the control sample with a compound or agent capable of detecting NT69 protein, and comparing the presence of NT69 protein in the control sample with the presence of NT69 protein in the test sample.

[00228] The invention also includes kits for detecting the presence of NT69 in a biological sample. For example, the kit can include a compound or agent capable of detecting NT69 protein or mRNA in a biological sample; and a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect NT69 protein or nucleic acid.

[00229] For antibody-based kits, the kit can include: (1) a first antibody (e.g., attached to a solid support) which binds to a polypeptide corresponding to a marker of the invention; and, optionally, (2) a second, different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable agent.

[00230] For oligonucleotide-based kits, the kit can include: (1) an oligonucleotide, e.g., a detectably labeled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide corresponding to a marker of the invention or (2) a pair of primers useful for amplifying a nucleic acid molecule corresponding to a marker of the invention. The kit can also includes a buffering agent, a preservative, or a protein stabilizing agent. The kit can also includes components necessary for detecting the detectable agent (e.g., an enzyme or a substrate). The kit can also contain a control sample or a series of control samples which can be assayed and compared to the test sample contained. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a

single package, along with instructions for interpreting the results of the assays performed using the kit.

[00231] The diagnostic methods described herein can identify subjects having, or at risk of developing, a disease or disorder associated with misexpressed or aberrant or unwanted NT69 expression or activity. As used herein, the term "unwanted" includes an unwanted phenomenon involved in a biological response such as pain or deregulated cell proliferation.

[00232] In one embodiment, a disease or disorder associated with aberrant or unwanted NT69 expression or activity is identified. A test sample is obtained from a subject and NT69 protein or nucleic acid (e.g., mRNA or genomic DNA) is evaluated, wherein the level, e.g., the presence or absence, of NT69 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant or unwanted NT69 expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest, including a biological fluid (e.g., serum), cell sample, or tissue.

[00233] The prognostic assays described herein can be used to determine whether a subject can be administered an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant or unwanted NT69 expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for obesity or an obesity related disorder.

[00234] The methods of the invention can also be used to detect genetic alterations in an NT69 gene, thereby determining if a subject with the altered gene is at risk for a disorder characterized by misregulation in NT69 protein activity or nucleic acid expression, such as obesity or pain. In preferred embodiments, the methods include detecting, in a sample from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding an NT69-protein, or the mis-expression of the NT69 gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from an NT69 gene; 2) an addition of one or more nucleotides to an NT69 gene; 3) a substitution of one or more nucleotides of an NT69 gene, 4) a chromosomal rearrangement of an NT69 gene; 5) an alteration in the level of a messenger RNA transcript of an NT69 gene, 6) aberrant modification of an NT69 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an NT69 gene,

8) a non-wild type level of an NT69-protein, 9) allelic loss of an NT69 gene, and 10) inappropriate post-translational modification of an NT69-protein.

[00235] An alteration can be detected without a probe/primer in a polymerase chain reaction, such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR), the latter of which can be particularly useful for detecting point mutations in the NT69-gene. This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (e.g., genomic, mRNA or both) from the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to an NT69 gene under conditions such that hybridization and amplification of the NT69-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

[00236] Alternative amplification methods include: self sustained sequence replication (Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) *Bio-Technology* 6:1197), or other nucleic acid amplification methods, followed by the detection of the amplified molecules using techniques known to those of skill in the art.

[00237] In another embodiment, mutations in an NT69 gene from a sample cell can be identified by detecting alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined, e.g., by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

[00238] In other embodiments, genetic mutations in NT69 can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, two dimensional arrays, e.g., chip based arrays. Such arrays include a plurality of addresses, each of which is positionally distinguishable from the other. A different probe is located at each address of the plurality. The arrays can have a high density of addresses, e.g., can contain hundreds or thousands of oligonucleotides probes (Cronin et al. (1996) *Human Mutation* 7:244-255; Kozal et al. (1996)

Nature Medicine 2:753-759). For example, genetic mutations in NT69 can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin et al., *supra.* Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

[00239] In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the NT69 gene and detect mutations by comparing the sequence of the sample NT69 with the corresponding wild-type (control) sequence. Automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry.

[00240] Other methods for detecting mutations in the NT69 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) *Science* 230:1242; Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba et al. (1992) *Methods Enzymol.* 217:286-295).

[00241] In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in NT69 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662; U.S. Patent No. 5,459,039).

[00242] In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in NT69 genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125-144; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control NT69 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids

varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) *Trends Genet* 7:5).

[00243] In yet another embodiment, the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

[00244] Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230).

[00245] Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

[00246] The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving an NT69 gene.

Use of NT69 Molecules as Surrogate Markers

[00247] The NT69 molecules of the invention are also useful as markers of disorders or disease states, as markers for precursors of disease states, as markers for predisposition of disease states, as markers of drug activity, or as markers of the pharmacogenomic profile of a subject. Using the methods described herein, the presence, absence and/or quantity of the NT69 molecules of the invention may be detected, and may be correlated with one or more biological states in vivo. For example, the NT69 molecules of the invention may serve as surrogate markers for one or more disorders or disease states or for conditions leading up to disease states. As used herein, a "surrogate marker" is an objective biochemical marker which correlates with the absence or presence of a disease or disorder, or with the progression of a disease or disorder (e.g., with the presence or absence of a tumor). The presence or quantity of such markers is independent of the disease. Therefore, these markers may serve to indicate whether a particular course of treatment is effective in lessening a disease state or disorder. Surrogate markers are of particular use when the presence or extent of a disease state or disorder is difficult to assess through standard methodologies (e.g., early stage tumors), or when an assessment of disease progression is desired before a potentially dangerous clinical endpoint is reached (e.g., an assessment of cardiovascular disease may be made using cholesterol levels as a surrogate marker, and an analysis of HIV infection may be made using HIV RNA levels as a surrogate marker, well in advance of the undesirable clinical outcomes of myocardial infarction or fully-developed AIDS). Examples of the use of surrogate markers in the art include: Koomen et al. (2000) *J. Mass. Spectrom.* 35: 258-264; and James (1994) AIDS Treatment News Archive 209.

[00248] The NT69 molecules of the invention are also useful as pharmacodynamic markers. As used herein, a "pharmacodynamic marker" is an objective biochemical marker which correlates specifically with drug effects. The presence or quantity of a pharmacodynamic marker is not related to the disease state or disorder for which the drug is being administered; therefore, the presence or quantity of the marker is indicative of the

presence or activity of the drug in a subject. For example, a pharmacodynamic marker may be indicative of the concentration of the drug in a biological tissue, in that the marker is either expressed or transcribed or not expressed or transcribed in that tissue in relationship to the level of the drug. In this fashion, the distribution or uptake of the drug may be monitored by the pharmacodynamic marker. Similarly, the presence or quantity of the pharmacodynamic marker may be related to the presence or quantity of the metabolic product of a drug, such that the presence or quantity of the marker is indicative of the relative breakdown rate of the drug in vivo. Pharmacodynamic markers are of particular use in increasing the sensitivity of detection of drug effects, particularly when the drug is administered in low doses. Since even a small amount of a drug may be sufficient to activate multiple rounds of marker (e.g., a NT69 marker) transcription or expression, the amplified marker may be in a quantity which is more readily detectable than the drug itself. Also, the marker may be more easily detected due to the nature of the marker itself; for example, using the methods described herein, anti-NT69 antibodies may be employed in an immune-based detection system for a NT69 protein marker, or NT69-specific radiolabeled probes may be used to detect a NT69 mRNA marker. Furthermore, the use of a pharmacodynamic marker may offer mechanism-based prediction of risk due to drug treatment beyond the range of possible direct observations. Examples of the use of pharmacodynamic markers in the art include: Matsuda et al. US 6,033,862; Hattis et al. (1991) *Env. Health Perspect.* 90: 229-238; Schentag (1999) *Am. J. Health-Syst. Pharm.* 56 Suppl. 3: S21-S24; and Nicolau (1999) Am. *J. Health-Syst. Pharm.* 56 Suppl. 3: S16-S20.

[00249] The NT69 molecules of the invention are also useful as pharmacogenomic markers. As used herein, a "pharmacogenomic marker" is an objective biochemical marker which correlates with a specific clinical drug response or susceptibility in a subject (see, e.g., McLeod et al. (1999) *Eur. J. Cancer* 35: 1650-1652). The presence or quantity of the pharmacogenomic marker is related to the predicted response of the subject to a specific drug or class of drugs prior to administration of the drug. By assessing the presence or quantity of one or more pharmacogenomic markers in a subject, a drug therapy which is most appropriate for the subject, or which is predicted to have a greater degree of success, may be selected. For example, based on the presence or quantity of RNA, or protein (e.g., NT69 protein or RNA) for specific tumor markers in a subject, a drug or course of treatment may be selected that is optimized for the treatment of the specific tumor likely to be present in the subject. Similarly, the presence or absence of a specific sequence mutation in NT69 DNA may correlate with NT69 drug response. The use of pharmacogenomic markers therefore

permits the application of the most appropriate treatment for each subject without having to administer the therapy.

Pharmaceutical Compositions

[00250] The nucleic acid and polypeptides, fragments thereof, as well as anti-NT69 antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions. Such compositions typically include the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

[00251] A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00252] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a

solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00253] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00254] Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[00255] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[00256] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[00257] The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[00258] In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

[00259] It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[00260] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and

therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit high therapeutic indeces are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00261] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00262] As defined herein, a therapeutically effective amount of protein or polypeptide (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The protein or polypeptide can be administered one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

[00263] For antibodies, the preferred dosage is 0.1 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower

dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. ((1997) J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:193).

[00264] The present invention encompasses agents which modulate expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e,. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[00265] Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

[00266] An antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Nonlimiting examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide,

vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin, and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

[00267] The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, .alpha.-interferon, .beta.-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophase colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

[00268] Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

[00269] The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

[00270] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

Methods of Treatment

[00271] The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant or unwanted NT69 expression or activity. With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers to the study of how a patient's genes determine his or her response to a drug (e.g., a patient's "drug response phenotype," or "drug response genotype"). Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the NT69 molecules of the present invention or NT69 modulators according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

[00272] "Treatment", or "treating a patient" or “treating a subject”, as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, palliate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. A therapeutic agent includes, but is not limited to, small molecules, peptides, antibodies, ribozymes and antisense oligonucleotides.

[00273] In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant or unwanted NT69 expression or activity, by administering to the subject an NT69 or an agent which modulates NT69 expression or at least one NT69 activity. Subjects at risk for a disease which is caused or contributed to by aberrant or unwanted NT69 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a

prophylactic agent can occur prior to the manifestation of symptoms characteristic of the NT69 aberrance, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of NT69 aberrance, for example, an NT69 nucleic acid or polypeptide, or a fragment thereof, NT69 agonist, or NT69 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

It is possible that some NT69 disorders can be caused, at least in part, by an abnormal level of gene product, or by the presence of a gene product exhibiting abnormal activity. As such, the reduction in the level and/or activity of such gene products would bring about the amelioration of disorder symptoms. For example, individuals subjected to an overfeeding regimen showed approximately 8-fold overexpression of NT69 mRNA in fat tissues compared to the same fat tissues before the overfeeding regimen (see Figure 4). Accordingly, modulating the expression of NT69 could be beneficial for those prone to obesity. The NT69 molecules can thus act as novel diagnostic targets and therapeutic agents for controlling obesity and other disorders of metabolic imbalance, which include, but are not limited to, anorexia nervosa, cachexia, lipid disorders (e.g., hyperlipidemia), and diabetes.

[00274] Aberrant expression and/or activity of NT69 molecules may mediate disorders associated with bone metabolism. "Bone metabolism" refers to direct or indirect effects in the formation or degeneration of bone structures, e.g., bone formation, bone resorption, etc., which may ultimately affect the concentrations in serum of calcium and phosphate. This term also includes activities mediated by NT69 molecules effects in bone cells, e.g. osteoclasts and osteoblasts, that may in turn result in bone formation and degeneration. For example, NT69 molecules may support different activities of bone resorbing osteoclasts such as the stimulation of differentiation of monocytes and mononuclear phagocytes into osteoclasts. Accordingly, NT69 molecules that modulate the production of bone cells can influence bone formation and degeneration, and thus may be used to treat bone disorders. Examples of such disorders include, but are not limited to, osteoporosis, osteodystrophy, osteomalacia, rickets, osteitis fibrosa cystica, renal osteodystrophy, osteosclerosis, anti-convulsant treatment, osteopenia, fibrogenesis-imperfecta ossium, secondary hyperparathyrodism, hypoparathyroidism, hyperparathyroidism, cirrhosis, obstructive jaundice, drug induced metabolism, medullary carcinoma, chronic renal disease, rickets, sarcoidosis, glucocorticoid antagonism, malabsorption syndrome, steatorrhea, tropical sprue, idiopathic hypercalcemia and milk fever.

[00275] The NT69 nucleic acid and protein of the invention can be used to treat and/or diagnose a variety of immune disorders. Examples of hematopoieitic disorders or diseases include, but are not limited to, autoimmune diseases (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions,leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis), graft-versus-host disease, cases of transplantation, and allergy such as, atopic allergy.

[00276] Disorders which may be treated or diagnosed by methods described herein include, but are not limited to, disorders associated with an accumulation in the liver of fibrous tissue, such as that resulting from an imbalance between production and degradation of the extracellular matrix accompanied by the collapse and condensation of preexisting fibers. The methods described herein can be used to diagnose or treat hepatocellular necrosis or injury induced by a wide variety of agents including processes which disturb homeostasis, such as an inflammatory process, tissue damage resulting from toxic injury or altered hepatic blood flow, and infections (e.g., bacterial, viral and parasitic). For example, the methods can be used for the early detection of hepatic injury, such as portal hypertension or hepatic fibrosis. In addition, the methods can be employed to detect liver fibrosis attributed to inborn errors of metabolsim, for example, fibrosis resulting from a storage disorder such as Gaucher's disease (lipid abnormalities) or a glycogen storage disease, A1-antitrypsin deficiency; a disorder mediating the accumulation (e.g., storage) of an exogenous substance, for example, hemochromatosis (iron-overload syndrome) and copper storage diseases (Wilson's disease), disorders resulting in the accumulation of a toxic metabolite (e.g., tyrosinemia, fructosemia and galactosemia) and peroxisomal disorders (e.g., Zellweger syndrome). Additionally, the methods described herein may be useful for the early detection

and treatment of liver injury associated with the administration of various chemicals or drugs, such as for example, methotrexate, isonizaid, oxyphenisatin, methyldopa, chlorpromazine, tolbutamide or alcohol, or which represents a hepatic manifestation of a vascular disorder such as obstruction of either the intrahepatic or extrahepatic bile flow or an alteration in hepatic circulation resulting, for example, from chronic heart failure, veno-occlusive disease, portal vein thrombosis or Budd-Chiari syndrome.

[00277] Disorders involving the muscle, include disorders of the skeletal muscle in states of altered insulin-stimulated glucose metabolism (e.g., NIDDM, IDDM, hyperglycaemia, and hyperlipidaemia). Additional examples of skeletal muscle disorders include Marfan syndrome and osteogenesis imperfecta, and rhabdomyosarcoma.

[00278] Disorders involving the colon include, but are not limited to, congenital anomalies, such as atresia and stenosis, Meckel diverticulum, congenital aganglionic megacolon-Hirschsprung disease; enterocolitis, such as diarrhea and dysentery, infectious enterocolitis, including viral gastroenteritis, bacterial enterocolitis, necrotizing enterocolitis, antibiotic-associated colitis (pseudomembranous colitis), and collagenous and lymphocytic colitis, miscellaneous intestinal inflammatory disorders, including parasites and protozoa, acquired immunodeficiency syndrome, transplantation, drug-induced intestinal injury, radiation enterocolitis, neutropenic colitis (typhlitis), and diversion colitis; idiopathic inflammatory bowel disease, such as Crohn disease and ulcerative colitis; tumors of the colon, such as non-neoplastic polyps, adenomas, familial syndromes, colorectal carcinogenesis, colorectal carcinoma, and carcinoid tumors.

[00279] As discussed, successful treatment of NT69 disorders can be brought about by techniques that serve to inhibit the expression or activity of target gene products. For example, compounds, e.g., an agent identified using an assays described above, that proves to exhibit negative modulatory activity, can be used in accordance with the invention to prevent and/or ameliorate symptoms of NT69 disorders. Such molecules can include, but are not limited to peptides, phosphopeptides, small organic or inorganic molecules, or antibodies (including, for example, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and Fab, $F(ab')_2$ and Fab expression library fragments, scFV molecules, and epitope-binding fragments thereof).

[00280] Further, antisense and ribozyme molecules that inhibit expression of the target gene can also be used in accordance with the invention to reduce the level of target gene expression, thus effectively reducing the level of target gene activity. Still further, triple helix

molecules can be utilized in reducing the level of target gene activity. Antisense, ribozyme and triple helix molecules are discussed above.

[00281] It is possible that the use of antisense, ribozyme, and/or triple helix molecules to reduce or inhibit mutant gene expression can also reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles, such that the concentration of normal target gene product present can be lower than is necessary for a normal phenotype. In such cases, nucleic acid molecules that encode and express target gene polypeptides exhibiting normal target gene activity can be introduced into cells via gene therapy method. Alternatively, in instances in that the target gene encodes an extracellular protein, it can be preferable to co-administer normal target gene protein into the cell or tissue in order to maintain the requisite level of cellular or tissue target gene activity.

[00282] Another method by which nucleic acid molecules may be utilized in treating or preventing a disease characterized by NT69 expression is through the use of aptamer molecules specific for NT69 protein. Aptamers are nucleic acid molecules having a tertiary structure which permits them to specifically bind to protein ligands (see, e.g., Osborne, et al. *Curr. Opin. Chem Biol.* 1997, 1(1): 5-9; and Patel, D.J. *Curr Opin Chem Biol* 1997 Jun;1(1):32-46). Since nucleic acid molecules may in many cases be more conveniently introduced into target cells than therapeutic protein molecules may be, aptamers offer a method by which NT69 protein activity may be specifically decreased without the introduction of drugs or other molecules which may have pluripotent effects.

[00283] Antibodies can be generated that are both specific for target gene product and that reduce target gene product activity. Such antibodies may, therefore, by administered in instances whereby negative modulatory techniques are appropriate for the treatment of NT69 disorders. For a description of antibodies, see the Antibody section above.

[00284] In circumstances wherein injection of an animal or a human subject with an NT69 protein or epitope for stimulating antibody production is harmful to the subject, it is possible to generate an immune response against NT69 through the use of anti-idiotypic antibodies (see, for example, Herlyn (1999) *Ann. Med.* 31:66-78; and Bhattacharya-Chatterjee and Foon (1998) *Cancer Treat. Res.* 94:51-68). If an anti-idiotypic antibody is introduced into a mammal or human subject, it should stimulate the production of anti-anti-idiotypic antibodies, which should be specific to the NT69 protein. Vaccines directed to a disease characterized by NT69 expression may also be generated in this fashion.

[00285] In instances where the target antigen is intracellular and whole antibodies are used, internalizing antibodies may be preferred. Lipofectin or liposomes can be used to deliver the antibody or a fragment of the Fab region that binds to the target antigen into cells. Where fragments of the antibody are used, the smallest inhibitory fragment that binds to the target antigen is preferred. For example, peptides having an amino acid sequence corresponding to the Fv region of the antibody can be used. Alternatively, single chain neutralizing antibodies that bind to intracellular target antigens can also be administered. Such single chain antibodies can be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population (see, e.g., Marasco et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893).

[00286] The identified compounds that inhibit target gene expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to prevent, treat or ameliorate NT69 disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the disorders.

[00287] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00288] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such

information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

[00289] Another example of determination of effective dose for an individual is the ability to directly assay levels of "free" and "bound" compound in the serum of the test subject. Such assays may utilize antibody mimics and/or "biosensors" that have been created through molecular imprinting techniques. The compound which is able to modulate NT69 activity is used as a template, or "imprinting molecule," to spatially organize polymerizable monomers prior to their polymerization with catalytic reagents. The subsequent removal of the imprinted molecule leaves a polymer matrix which contains a repeated "negative image" of the compound and is able to selectively rebind the molecule under biological assay conditions. A detailed review of this technique can be seen in Ansell, R. J. *et al* (1996) *Current Opinion in Biotechnology* 7:89-94 and in Shea, K.J. (1994) *Trends in Polymer Science* 2:166-173. Such "imprinted" affinity matrixes are amenable to ligand-binding assays, whereby the immobilized monoclonal antibody component is replaced by an appropriately imprinted matrix. An example of the use of such matrixes in this way can be seen in Vlatakis, G. *et al* (1993) *Nature* 361:645-647. Through the use of isotope-labeling, the "free" concentration of compound which modulates the expression or activity of NT69 can be readily monitored and used in calculations of IC_{50}.

[00290] Such "imprinted" affinity matrixes can also be designed to include fluorescent groups whose photon-emitting properties measurably change upon local and selective binding of target compound. These changes can be readily assayed in real time using appropriate fiberoptic devices, in turn allowing the dose in a test subject to be quickly optimized based on its individual IC_{50}. An rudimentary example of such a "biosensor" is discussed in Kriz, D. *et al* (1995) *Analytical Chemistry* 67:2142-2144.

[00291] Another aspect of the invention pertains to methods of modulating NT69 expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with an NT69 or agent that modulates one or more of the activities of NT69 protein activity associated with the cell. An agent that modulates NT69 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring target molecule of an NT69 protein (e.g., an NT69 substrate or receptor), an NT69 antibody, an NT69 agonist or antagonist, a peptidomimetic of an NT69 agonist or antagonist, or other small molecule.

[00292] In one embodiment, the agent stimulates one or more NT69 activities. Examples of such stimulatory agents include active NT69 protein and a nucleic acid molecule encoding NT69. In another embodiment, the agent inhibits one or more NT69 activities. Examples of such inhibitory agents include antisense NT69 nucleic acid molecules, antiNT69 antibodies, and NT69 inhibitors. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of an NT69 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (e.g., an agent identified by a screening assay described herein), or combination of agents that modulates (e.g., upregulates or downregulates) NT69 expression or activity. In another embodiment, the method involves administering an NT69 protein or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted NT69 expression or activity.

[00293] Stimulation of NT69 activity is desirable in situations in which NT69 is abnormally downregulated and/or in which increased NT69 activity is likely to have a beneficial effect. For example, stimulation of NT69 activity is desirable in situations in which an NT69 is downregulated and/or in which increased NT69 activity is likely to have a beneficial effect. Likewise, inhibition of NT69 activity is desirable in situations in which NT69 is abnormally upregulated and/or in which decreased NT69 activity is likely to have a beneficial effect, e.g., in treating obesity.

Pharmacogenomics

[00294] The NT69 molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on NT69 activity (e.g., NT69 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) NT69 associated disorders (e.g., obesity and hyperlipidemia) associated with aberrant or unwanted NT69 activity. In conjunction with such treatment, pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant

pharmacogenomics studies in determining whether to administer an NT69 molecule or NT69 modulator as well as tailoring the dosage and/or therapeutic regimen of treatment with an NT69 molecule or NT69 modulator.

[00295] Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. et al. (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11):983-985 and Linder, M.W. et al. (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

[00296] One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association," relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (e.g., a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants.) Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

[00297] Alternatively, a method termed the "candidate gene approach", can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drug's target is known (e.g., an NT69 protein of the present invention), all common variants of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

[00298] Alternatively, a method termed the "gene expression profiling", can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (e.g., an NT69 molecule or NT69 modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

[00299] Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment of an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an NT69 molecule or NT69 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

[00300] The present invention further provides methods for identifying new agents, or combinations, that are based on identifying agents that modulate the activity of one or more of the gene products encoded by one or more of the NT69 genes of the present invention, wherein these products may be associated with resistance of the cells to a therapeutic agent. Specifically, the activity of the proteins encoded by the NT69 genes of the present invention can be used as a basis for identifying agents for overcoming agent resistance. By blocking the activity of one or more of the resistance proteins, target cells will become sensitive to treatment with an agent that the unmodified target cells were resistant to.

[00301] Monitoring the influence of agents (e.g., drugs) on the expression or activity of an NT69 protein can be applied in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase NT69 gene expression, protein levels, or upregulate NT69 activity, can be monitored in clinical trials of subjects exhibiting decreased NT69 gene expression, protein levels, or downregulated NT69 activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease NT69 gene expression, protein levels, or downregulate NT69 activity, can be monitored in clinical trials of subjects exhibiting increased NT69 gene expression, protein levels, or upregulated NT69 activity. In such clinical trials, the expression or activity of an NT69 gene,

and preferably, other genes that have been implicated in, for example, an NT69-associated disorder can be used as a "read out" or markers of the phenotype of a particular cell.

Other Embodiments

[00302] In another aspect, the invention features, a method of analyzing a plurality of capture probes. The method can be used, e.g., to analyze gene expression. The method includes: providing a two dimensional array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality, and each address of the plurality having a unique capture probe, e.g., a nucleic acid or peptide sequence; contacting the array with an NT69, preferably purified, nucleic acid, preferably purified, polypeptide, preferably purified, or antibody, and thereby evaluating the plurality of capture probes. Binding, e.g., in the case of a nucleic acid, hybridization with a capture probe at an address of the plurality, is detected, e.g., by signal generated from a label attached to the NT69 nucleic acid, polypeptide, or antibody.

[00303] The capture probes can be a set of nucleic acids from a selected sample, e.g., a sample of nucleic acids derived from a control or non-stimulated tissue or cell.

[00304] The method can include contacting the NT69 nucleic acid, polypeptide, or antibody with a first array having a plurality of capture probes and a second array having a different plurality of capture probes. The results of each hybridization can be compared, e.g., to analyze differences in expression between a first and second sample. The first plurality of capture probes can be from a control sample, e.g., a wild type, normal, or non-diseased, non-stimulated, sample, e.g., a biological fluid, tissue, or cell sample. The second plurality of capture probes can be from an experimental sample, e.g., a mutant type, at risk, disease-state or disorder-state, or stimulated, sample, e.g., a biological fluid, tissue, or cell sample.

[00305] The plurality of capture probes can be a plurality of nucleic acid probes each of which specifically hybridizes, with an allele of NT69. Such methods can be used to diagnose a subject, e.g., to evaluate risk for a disease or disorder, to evaluate suitability of a selected treatment for a subject, to evaluate whether a subject has a disease or disorder. NT69 is associated with faulty glycolytic control, thus it is useful for evaluating hemolytic anemia and diabetes.

[00306] The method can be used to detect SNPs, as described above.

[00307] In another aspect, the invention features, a method of analyzing a plurality of probes. The method is useful, e.g., for analyzing gene expression. The method includes:

providing a two dimensional array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality having a unique capture probe, e.g., wherein the capture probes are from a cell or subject which express NT69 or from a cell or subject in which an NT69 mediated response has been elicited, e.g., by contact of the cell with NT69 nucleic acid or protein, or administration to the cell or subject NT69 nucleic acid or protein; contacting the array with one or more inquiry probe, wherein an inquiry probe can be a nucleic acid, polypeptide, or antibody (which is preferably other than NT69 nucleic acid, polypeptide, or antibody); providing a two dimensional array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality, and each address of the plurality having a unique capture probe, e.g., wherein the capture probes are from a cell or subject which does not express NT69 (or does not express as highly as in the case of the NT69 positive plurality of capture probes) or from a cell or subject which in which an NT69 mediated response has not been elicited (or has been elicited to a lesser extent than in the first sample); contacting the array with one or more inquiry probes (which is preferably other than an NT69 nucleic acid, polypeptide, or antibody), and thereby evaluating the plurality of capture probes. Binding, e.g., in the case of a nucleic acid, hybridization with a capture probe at an address of the plurality, is detected, e.g., by signal generated from a label attached to the nucleic acid, polypeptide, or antibody.

[00308] In another aspect, the invention features, a method of analyzing a plurality of probes or a sample. The method is useful, e.g., for analyzing gene expression. The method includes: providing a two dimensional array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality having a unique capture probe, contacting the array with a first sample from a cell or subject which express or mis-express NT69 or from a cell or subject in which an NT69 mediated response has been elicited, e.g., by contact of the cell with NT69 nucleic acid or protein, or administration to the cell or subject NT69 nucleic acid or protein; providing a two dimensional array having a plurality of addresses, each address of the plurality being positionally distinguishable from each other address of the plurality, and each address of the plurality having a unique capture probe, and contacting the array with a second sample from a cell or subject which does not express NT69 (or does not express as highly as in the case of the NT69 positive plurality of capture probes) or from a cell or subject which in which an NT69 mediated response has not been elicited (or has been elicited to a lesser extent than in

the first sample); and comparing the binding of the first sample with the binding of the second sample. Binding, e.g., in the case of a nucleic acid, hybridization with a capture probe at an address of the plurality, is detected, e.g., by signal generated from a label attached to the nucleic acid, polypeptide, or antibody. The same array can be used for both samples or different arrays can be used. If different arrays are used the plurality of addresses with capture probes should be present on both arrays.

[00309] In another aspect, the invention features, a method of analyzing NT69, e.g., analyzing structure, function, or relatedness to other nucleic acid or amino acid sequences. The method includes: providing an NT69 nucleic acid or amino acid sequence; comparing the NT69 sequence with one or more preferably a plurality of sequences from a collection of sequences, e.g., a nucleic acid or protein sequence database; to thereby analyze NT69.

[00310] Preferred databases include PFAM, BLAST, ProDom and SMART. The method can include evaluating the sequence identity between an NT69 sequence and a database sequence. The method can be performed by accessing the database at a second site, e.g., over the internet.

[00311] In another aspect, the invention features, a set of oligonucleotides, useful, e.g., for identifying SNP's, or identifying specific alleles of NT69. The set includes a plurality of oligonucleotides, each of which has a different nucleotide at an interrogation position, e.g., an SNP or the site of a mutation. In a preferred embodiment, the oligonucleotides of the plurality identical in sequence with one another (except for differences in length). The oligonucleotides can be provided with diferential labels, such that an oligonucleotides which hybridizes to one allele provides a signal that is distinguishable from an oligonucleotides which hybridizes to a second allele.

[00312] The sequence of an NT69 molecules is provided in a variety of mediums to facilitate use thereof. A sequence can be provided as a manufacture, other than an isolated nucleic acid or amino acid molecule, which contains an NT69. Such a manufacture can provide a nucleotide or amino acid sequence, e.g., an open reading frame, in a form which allows examination of the manufacture using means not directly applicable to examining the nucleotide or amino acid sequences, or a subset thereof, as they exists in nature or in purified form.

[00313] AN NT69 nucleotide or amino acid sequence can be recorded on computer readable media. As used herein, "computer readable media" refers to any medium that can be read and accessed directly by a computer. Such media include, but are not limited to:

magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media.

[00314] A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide or amino acid sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. The skilled artisan can readily adapt any number of data processor structuring formats (*e.g.*, text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention.

[00315] By providing the nucleotide or amino acid sequences of the invention in computer readable form, the skilled artisan can routinely access the sequence information for a variety of purposes. For example, one skilled in the art can use the nucleotide or amino acid sequences of the invention in computer readable form to compare a target sequence or target structural motif with the sequence information stored within the data storage means. A search is used to identify fragments or regions of the sequences of the invention which match a particular target sequence or target motif.

[00316] As used herein, a "target sequence" can be any DNA or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. Typical sequence lengths of a target sequence are from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

[00317] Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium for analysis and comparison to other sequences. A variety of known algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be used in the

computer-based systems of the present invention. Examples of such software include, but are not limited to, MacPattern (EMBL), BLASTN and BLASTX (NCBIA).

[00318] Thus, the invention features a method of making a computer readable record of a sequence of an NT69 sequence which includes recording the sequence on a computer readable matrix. In a preferred embodiment the record includes one or more of the following: identification of an ORF; identification of a domain, region, or site; identification of the start of transcription; identification of the transcription terminator; the full length amino acid sequence of the protein, or a mature form thereof; the 5' end of the translated region.

[00319] In another aspect, the invention features, a method of analysing a sequence. The method includes: providing an NT69 sequence,or record, in computer readable form; comparing a second sequence to the gene name sequence; thereby analysing a sequence. Comparison can include comparing to sequences for sequence identity or determining if one sequence is included within the other, e.g., determining if the NT69 sequence includes a sequence being compared. In a preferred embodiment the NT69 or second sequence is stored on a first computer, e.g., at a first site and the comparison is performed, read, or recorded on a second computer, e.g., at a second site. E.g., the NT69 or second sequence can be stored in a public or proprietary database in one computer, and the results of the comparison performed, read, or recorded on a second computer. In a preferred embodiment the record includes one or more of the following: identification of an ORF; identification of a domain, region, or site; identification of the start of transcription; identification of the transcription terminator; the full length amino acid sequence of the protein, or a mature form thereof; the 5' end of the translated region.

[00320] This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example 1: Identification and Characterization of Human NT69 cDNA

[00321] NT69 was first identified as a 469 base-pair EST sequence from a human islet library. DNA hybridization analysis with the EST sequence was used to identify a novel, full-length gene, NT69.

[00322] The human NT69 sequence (Fig. 1; SEQ ID NO:1), which is approximately 2625 nucleotides long, including untranslated regions, contains a predicted methionine-initiated coding sequence of about 1425 nucleotides (nucleotides indicated as "coding" of SEQ ID NO:1 in Fig. 1; SEQ ID NO:3). The coding sequence encodes a 475 amino acid protein (SEQ ID NO:2).

<u>Example 2: Tissue Distribution of NT69 mRNA</u>

[00323] Total RNA was prepared from various mouse tissues according to the manufacturer's instructions (Gibco BRL). Each RNA preparation was treated with DNase I (Gibco BRL) at room temperature for 15 min. cDNA was prepared from the sample using the SUPERSCRIPT™ First-Strand Synthesis System for RT-PCR following the manufacturer's instructions (GibcoBRL). A negative control of RNA without reverse transcriptase was mock reverse transcribed for each RNA sample.

[00324] Novel NT69 expression was measured by TaqMan® quantitative PCR (Applied Biosystems) in cDNA prepared from the following human tissues: Artery, Vein, Aortic SMC EARLY, Coronary SMC, Static HUVEC, Shear HUVEC, Heart, Heart CHF, Kidney, Skeletal Muscle, Adipose, Pancreas, primary osteoblasts, Osteoclasts (diff), Skin, Spinal cord, Brain Cortex, Brain Hypothalamus, Nerve, DRG (Dorsal Root Ganglion),

[00325] Glial Cells, Glioblastoma (Astrocytes), Breast, Breast tumor, Ovary, Ovary Tumor, Prostate, Prostate Tumor, Epithelial Cells (Prostate), Colon, Colon Tumor, Lung, Lung tumor, Lung COPD, Colon IBD, Liver, Dermal Cells-fibroblasts, Spleen, Tonsil, Lymph node, Skin-Decubitus, Synovium, BM-MNC (Bone marrow mononuclear cells) from one or two adult donors; fibrotic liver samples prepared from two to seven different donors; resting and phytohemaglutinin-activated peripheral blood mononuclear cells (PBMC).

[00326] PCR probes were designed by PrimerExpress software (Applied Biosystems) based on the disclosed sequences of novel human and mouse NT69 gene. Primers and probes for expression analysis of β-2 microglobulin and 18s rRNA were also designed as controls.

[00327] Each NT69 gene probe was labeled using FAM (6-carboxyfluorescein), but the 18s rRNA and β 2 -microglobulin reference probes were labeled with a different fluorescent dye, VIC. The differential labeling of the target gene and internal reference gene thus enabled measurement in the same well. Forward and reverse primers and the probes for 18s or β2-microglobulin and target gene were added to the TaqMan® Universal PCR Master Mix

(Applied Biosystems). Although the final concentration of primer and probe could vary, each was internally consistent within a given experiment.

[00328] A typical experiment contained 1X 18s rRNA control mix, 200 nM of forward and reverse primers plus 100nM probe for β-2 microglobulin and 600 nM forward and reverse primers plus 200 nM probe for the target gene. TaqMan matrix experiments were carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The thermal cycler conditions were as follows: hold for 2 min at 50°C and 10 min at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 sec followed by 60°C for 1 min.

[00329] The following method was used to quantitatively calculate NT69 gene expression in the various tissues relative to 18s rRNA or β-2 microglobulin expression in the same tissue. The threshold cycle (Ct) value is defined as the cycle at which a statistically significant increase in fluorescence is detected. A lower Ct value is indicative of a higher mRNA concentration. The Ct value of the NT69 gene is normalized by subtracting the Ct value of the 18s rRNA or β-2 microglobulin gene to obtain a ${}_{\Delta}Ct$ value using the following formula: ${}_{\Delta}Ct=Ct_{NT69} - Ct_{18s\ rRNA}$, or ${}_{\Delta}Ct=Ct_{NT69}-Ct_{b2\text{-}microglobulin}$. Expression is then calibrated against a cDNA sample showing a comparatively higher level expression of the NT69 gene in human fat tissues, but lower level expression of the NT69 gene in mouse BAT and WAT. The ${}_{\Delta}Ct$ value for the calibrator sample is then subtracted from ${}_{\Delta}Ct$ for each tissue sample according to the following formula: ${}_{\Delta\Delta}Ct = {}_{\Delta}Ct\text{-}_{sample} - {}_{\Delta}Ct\text{-}_{calibrator}$. Relative expression is then calculated using the arithmetic formula given by $2^{-\Delta\Delta Ct}$ Expression of the target NT69 gene in each of the tissues tested is then graphically represented as discussed in more detail below.

[00330] NT69 expression is highest in human brain cortex, hypothalamus, glial cells, and fat tissue. It is also present in other tissues including epithelial cells, kidney, pancreas, and lymph node (Figure 4). No measurable expression was observed in, e.g., artery, vein, skeletal muscle, primary osteoblasts, osteoclasts, nerve, breast, ovary, lung, spleen, tonsil, peripheral blood mononuclear cells, skin-decubitus, and synovium (Figure 4).

<u>Example 3: Recombinant Expression of NT69 in Bacterial Cells</u>

[00331] In this example, NT69 is expressed as a recombinant glutathione-S-transferase (GST) fusion polypeptide in *E. coli* and the fusion polypeptide is isolated and characterized. Specifically, NT69 is fused to GST and this fusion polypeptide is expressed in *E. coli*, e.g., strain PEB199. Expression of the GST-NT69 fusion protein in PEB199 is induced with IPTG. The recombinant fusion polypeptide is purified from crude bacterial lysates of the

induced PEB199 strain by affinity chromatography on glutathione beads. Using polyacrylamide gel electrophoretic analysis of the polypeptide purified from the bacterial lysates, the molecular weight of the resultant fusion polypeptide is determined.

Example 4: Expression of Recombinant NT69 Protein in COS Cells

[00332] To express the NT69 gene in COS cells, the pcDNA/Amp vector by Invitrogen Corporation (San Diego, CA) is used. This vector contains an SV40 origin of replication, an ampicillin resistance gene, an *E. coli* replication origin, a CMV promoter followed by a polylinker region, and an SV40 intron and polyadenylation site. A DNA fragment encoding the entire NT69 protein and an HA tag (Wilson et al. (1984) *Cell* 37:767) or a FLAG tag fused in-frame to its 3' end of the fragment is cloned into the polylinker region of the vector, thereby placing the expression of the recombinant protein under the control of the CMV promoter.

[00333] To construct the plasmid, the NT69 DNA sequence is amplified by PCR using two primers. The 5' primer contains the restriction site of interest followed by approximately twenty nucleotides of the NT69 coding sequence starting from the initiation codon; the 3' end sequence contains complementary sequences to the other restriction site of interest, a translation stop codon, the HA tag or FLAG tag and the last 20 nucleotides of the NT69 coding sequence. The PCR amplified fragment and the pCDNA/Amp vector are digested with the appropriate restriction enzymes and the vector is dephosphorylated using the CIAP enzyme (New England Biolabs, Beverly, MA). Preferably the two restriction sites chosen are different so that the NT69_gene is inserted in the correct orientation. The ligation mixture is transformed into *E. coli* cells (strains HB101, DH5α, SURE, available from Stratagene Cloning Systems, La Jolla, CA, can be used), the transformed culture is plated on ampicillin media plates, and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment.

[00334] COS cells are subsequently transfected with the NT69-pcDNA/Amp plasmid DNA using the calcium phosphate or calcium chloride co-precipitation methods, DEAE-dextran-mediated transfection, lipofection, or electroporation. Other suitable methods for transfecting host cells can be found in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The expression of the NT69 polypeptide is detected by radiolabelling (^{35}S-methionine or ^{35}S-cysteine available from

NEN, Boston, MA, can be used) and immunoprecipitation (Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) using an HA specific monoclonal antibody. Briefly, the cells are labeled for 8 hours with ^{35}S-methionine (or ^{35}S-cysteine). The culture media are then collected and the cells are lysed using detergents (RIPA buffer, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5). Both the cell lysate and the culture media are precipitated with an HA specific monoclonal antibody. Precipitated polypeptides are then analyzed by SDS-PAGE.

[00335] Alternatively, DNA containing the NT69 coding sequence is cloned directly into the polylinker of the pCDNA/Amp vector using the appropriate restriction sites. The resulting plasmid is transfected into COS cells in the manner described above, and the expression of the NT69 polypeptide is detected by radiolabelling and immunoprecipitation using an NT69 specific monoclonal antibody.

Example 5: NT69 activity assays

[00336] The functional properties of NT69 are investigated by expressing human NT69 in *Xenopus* oocytes, which lack endogenous nucleoside transport activity. Nucleoside uptake assays are performed in *Xenopus* oocytes as described in Griffiths et al. (1997) *Nature Med* 3:89-93.

Example 6: Expression of NT69 mRNA in Rat Models of Pain

[00337] Expression of NT69 was also examined in a panel of normal rat tissues using the RNA preparation method and analysis by TaqMan® quantitative PCR as described above in Example 2. The results indicated that rat NT69 was expressed in the following normal neurological tissues: superior cervical ganglion, which showed a significantly high level of expression, and brain, optic nerve, spinal cord, and dorsal root ganglion, all of which showed comparable, but lower levels of expression. NT69 was also detectable at low levels in normal rat adrenal gland, thyroid, and thymus tissues.

[00338] The expression of NT69 in these normal neurological tissues led to studies of NT69 expression in animal models of pain. In these models, rats were subjected to the following procedures: ligation of the sciatic nerve to produce chronic constriction injury (Bennett GJ & Xie YK, 1988; Pain 33:87-107), plantar injection of complete Freund's adjuvant (Stein C, Millan MJ and Herz A, 1988; Pharmacol. Biochem Behav 31; 445-451) to

produce inflammatory pain, or axotomy of the sciatic nerve (Curtis et al., 1994; Neuron 12; 191-204) to produce chronic pain. Total RNA from spinal cord and dorsal root ganglia was prepared by a single step extraction method using RNA STAT-60 according to the manufacturer's instructions (TelTest, Inc). Each RNA preparation was treated with DNase I (Ambion) at 37°C for 1 hour. DNAse I treatment was determined to be complete if the sample required at least 38 PCR amplification cycles to reach a threshold level of fluorescence using 18S as an internal amplicon reference. The integrity of the RNA samples following DNase I treatment was confirmed by 1.2% agarose gel electrophoresis. After phenol extraction cDNA was prepared from the sample using the TaqMan® reverse transcription reagent (PE Applied Biosystems). A negative control of RNA without reverse transcriptase was mock reverse transcribed for each RNA sample.

[00339] Expression of the rat ortholog of human NT69 was measured by TaqMan® quantitative PCR (PE Applied Biosystems) RNA prepared from dorsal root ganglion (DRG) and spinal cord (SC) using the procedure essentially described above in Example 2.

[00340] DRG tissue samples were collected from three rats in each model of pain one, three, seven, ten, fourteen, and twenty-eight days after surgery or treatment. The results summarized in Figure 5 show that NT69 expression in the CCI and AXT models of pain was higher than in DRG from naïve control rats. Furthermore, the increase in NT69 expression in these models continued over time. The pattern of NT69 expression in DRG taken from rats in the inflammatory pain model did not show either a higher level of expression as compared to nontreated rats or an increase in expression over time.

[00341] Figure 6 summarizes the pattern of expression of NT69 in spinal cord (SC) RNA obtained from the same rat models of pain described for the results summarized in Figure 5. As shown in Figure 6, the expression of NT69 in all three models of pain were higher than for the SC sample taken from the corresponding naïve control rats. Additionally, all three models present a tendency towards increased NT69 expression over time.

[00342] In situ hybridization experiments with both rat and mouse tissues showed that expression of NT69 is present in regions of the central and peripheral nervous systems that are involved in pain processing. For example, NT69 expression was detected in the cingulate cortex, thalamus, spinal cord and subpopulation of dorsal root ganglion neurons including small diameter neurons.

[00343] Taken collectively, these expression data demonstrate a role for NT69 in pain.

Equivalents

[00344] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.